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STUDIES ON THE MECHANISM OF SHOCK. THE EFFECT OF LIMB ISCHAEMIA ON THE PHOSPHATES OF MUSCLE

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THE fact that limb ischaemia is followed by a general reaction of the body—shock—which may lead to death has aroused much interest in the chemical changes in the damaged muscle but knowledge of these changes is still incomplete because of the difficulty of measuring them accurately.

Using a biological assay, Billings and Maegraith (1937) reported an increase in the " adenosine equivalent " of extracts from partially ischaemic rabbit muscle but it was found that more complete ischaemia greatly reduced the level of adenyl compounds in the muscle (Green, 1943 ; Bielschowsky, 1947 ; Green, Bielschowsky and Stoner, 1947).

Bollman and Flock (1944) made an important contribution when they showed chemically that after 1 hr. period of ischaemia the phosphocreatine (PC) had almost completely disappeared followed by the loss of acid-labile P (mostly from adenosine triphosphate—ATP) after a further 2 hr. ischaemia. These changes were accompanied by a rise in the inorganic phosphate fraction of the muscle while the total acid-soluble phosphate content remained unchanged. What happened when the circulation was restored to the ischaemic muscle depended on the duration of the ischaemia. Acid-labile P and PC were only resynthesized if this was less than 3 hr.

These results have been essentially confirmed by LePage (1946), Macfarlane and Spooner (1946), Goranson, Hamilton and Haist (1948), Harman and Gwinn (1949), Kovách *et al.* (1952), Takács, Kovách, Takács-Nagy and Szabo (1954) and Tremolières, Derache and Griffaton (1955). Goranson *et al.* showed that these changes in the damaged muscle were not reflected in the other muscles. This was at variance with the findings of LePage and of Kovách *et al.* who considered that there was a generalized reduction in these muscle constituents in fatal shock. We have confirmed the results of Goranson *et al.*, finding no change in the PC and ATP levels of the undamaged muscle until just before death when there was

moderate fall in the PC content (Stoner and Threlfall, 1954). Regarding ATP resynthesis, in the experiments of Harman and Gwinn it occurred despite a 4 hr. period of ischaemia and Bielschowsky and Green (1945) have shown that enzyme systems exist in the damaged muscle which are capable of performing this synthesis.

Despite the apparent abundance of information on the changes in muscle phosphates during and after ischaemia they still cannot be accurately described. This is because the methods depended on graded acid hydrolysis and barium fractionation and lacked specificity. An attempt to overcome this difficulty has recently been made by Jordan and Gray (1955) using ion-exchange columns, and in our laboratory a method using charcoal adsorption and chromatography has been devised (Threlfall, 1957) for estimating all the P-containing components of an acid extract of muscle. The present paper will be confined to the changes in the PC, nucleotide and coenzyme I concentrations in muscle; the effect of limb ischaemia on the ester phosphates of the glycolytic cycle will be described later.

METHODS

The experiments were performed on 206 albino rats of the Porton strain (body wt. 212 ± 1·7 g.) fed on M.R.C. diet 41 (Bruce and Parkes, 1949). The rats, except those acclimatized to cold, were kept in the laboratory for about a week before the experiment to accustom them to their surroundings. During this period they were given food and water *ad lib.* At the start of the experiment, food, but not water, was removed. Hind-limb ischaemia was produced with rubber tourniquets (Rosenthal, 1943) applied during ether anaesthesia of about 3 min. duration. Control rats were given a similar period of ether anaesthesia. The experiments were usually carried out at an environmental temperature of 18–22°. In some cases this was raised to 30° by putting the animals in a thermostatically controlled cabinet. Some of the rats were acclimatized to cold by keeping them at 2–3° for 58–74 days before carrying out the experiments at an air temperature of 18–22°.

The blood supply to the injured limbs was assessed by methods used previously (Stoner and Green, 1948; Stoner, 1954).

Plasma samples were obtained from blood withdrawn with a syringe from the abdominal aorta or inferior vena cava, the rat being anaesthetized with Na pentobarbitone (Veterinary Nembutal-Abbott). Heparin was used as an anticoagulant.

Muscle samples were obtained from the fore- and hind-limbs as previously described (Stoner and Threlfall, 1954). The phosphate fractions in 3 per cent perchloric acid extracts of these muscles were determined as described by Threlfall (1957). The water content of the muscle was determined by drying samples to a constant weight at 105°.

Radioactive phosphate was obtained from the Radiochemical Centre, Amersham, as a solution in sterile phosphate-saline buffer (pH 7·0; 1 mg. P per ml.). The solutions were standardized as before (Stoner, Threlfall and Green, 1952) and injected intramuscularly (fore-limb) in a dose of 50 μc ^{32}P per 100 g. body wt. The hind-limbs were perfused for 4 min. through the abdominal aorta with ice-cold Krebs-Henseleit (1932) Ringer's solution at a pressure of 100 mm. Hg before excising the muscle samples. The radioactivity of nucleotide fractions on the one-dimensional chromatograms was assessed by cutting them into strips which were passed cm. by cm. beneath an end-window counter (Type EHM2/S, General Electric Co.) attached to conventional scaling apparatus. Their activity on the two-dimensional chromatograms was assessed with an end-window counter (Type EW–3H, 20th Century Electronics) attached to an Airmec Radiation monitor.

When possible the results are expressed as the mean ± standard error and where necessary a statistical comparison of the means has been done by Student's "*t*" test as modified by Fisher (1934) for small samples.

The abbreviations used in this paper are as follows: Phosphocreatine, PC; adenosine triphosphate, diphosphate and monophosphate, ATP, ADP, and AMP; inosine monophosphate, IMP; guanosine triphosphate, GTP; uridine triphosphate UTP; diphosphopyridine nucleotide, oxidized form DPN, reduced form DPNH; energy-rich phosphate, ~ P, *i.e.*, the energy associated with the pyrophosphate linkage in nucleoside di- and triphosphates and with the phospho-amide linkage in phosphocreatine (Lipmann, 1941).

RESULTS

General effects of limb ischaemia

The mortality rates and survival times after different periods of ischaemia at an air temperature of 18–22° in parallel series of rats are shown in Table I. This Table also shows the effect of increasing the environmental temperature to 30° and the effect of cold-acclimatization on the resistance of rats to 4 hr. bilateral hind-limb ischaemia at an environmental temperature of 18–22°. The increased resistance of the cold-acclimatized rat has been reported by Haist, Schachter, Sidlofsky, Hamilton and Baker (1956).

TABLE I.—*The Mortality Rate and Average Survival Time after Different Periods of Bilateral Hind-limb Ischaemia in Normal and Cold-acclimatized rats*

Animal	Number of rats	Environmental temperature°	Period of ischaemia (hr.)	Mortality rate (per cent)	Survival time (hr.) (mean ± S.E.)
Normal rats	—	18–22	2	0	—
" "	119	18–22	4	85	13·28±0·90
" "	23	18–22	6	100	7·70±0·88
" "	33	30	4	100	2·22±0·32
Cold-acclimatized rats	13	18–22	4	23	<20

Blood flow to the hind-limbs during and after ischaemia

When 0·5 ml. 20 per cent Na fluoresceinate was injected intraperitoneally 30 min. after the tourniquets had been applied and the rats killed at the end of 4 hr. with the tourniquets still in place, the fluorescence of the skinned limbs in ultra-violet light ended abruptly at the tourniquets in all but 2 of the 18 rats. In the 2 exceptions there were some small patches of slight staining in the muscles below the tourniquets. When fluorescein was given immediately after removal of the tourniquets uniform fluorescence of the hind-limb muscles was produced.

In 7 rats 0·5 mg. strychnine nitrate per 100 g. body wt. (approx. 10 M.L.D.) was injected into the muscles below the tourniquet. No symptoms appeared while the tourniquets were in place but when they were removed after 4 hr., signs of poisoning appeared in 2–5 min. and all animals were dead in 10 min. The survival time after this dose of strychnine was about the same in the controls.

More quantitative data were obtained by observing the muscle temperature in the soleus or under surface of the gastrocnemius muscles. With an air temperature of 18–22° the temperature fell rapidly after applying the tourniquets (Fig. 1A); indeed for the first 2 hr. the rate of fall was about the same as in muscle autolysing *in situ*. Subsequently the temperature of the autolytic muscle fell below that of the ischaemic muscle. The average temperature of the latter after 4 hr. ischaemia was 23·3° ± 0·5 (19 rats). At this time the temperature of the autolytic muscle was the same as that of the surrounding air and the higher temperature of the ischaemic muscle was probably due to conduction of heat from the body of the rat. There was no tendency for the muscle temperature to rise towards the end of the ischaemic period (Stoner and Green, 1948) and the tourniquets appeared to cause complete ischaemia of the limb. When they were applied at an air temperature of 30° the muscle temperature quickly fell to an average of 32·3° ± 0·4 (5 rats) where it remained for the rest of the ischaemic period (Fig. 2).

Removal of the tourniquets was followed by a rise in muscle temperature. After 4 hr. bilateral hind-limb ischaemia at an environmental temperature of

18–22°, it rose to 34·4° ± 0·4 (17 rats). The peak was usually reached in the first 30 min. after return of the circulation. This increase was short-lived for after 1 hr. the muscle temperature fell progressively until the rat died (Fig. 1A). The changes in the cold-acclimatized rats were the same. After a 4 hr. period of bilateral hind-limb ischaemia the muscle temperature was 24·8° ± 1·0 (6 rats) and when the tourniquets were removed it rose to 35·2° ± 0·7 (4 rats.) This rise was better maintained in the cold-acclimatized rats.

After 2 hr. ischaemia (Fig. 1B) the muscle temperature was restored more closely to the initial level, reaching 35·8° ± 0·4 (4 rats) and the secondary fall was less marked. After 6 hr. ischaemia the rise in temperature in the hind-limb

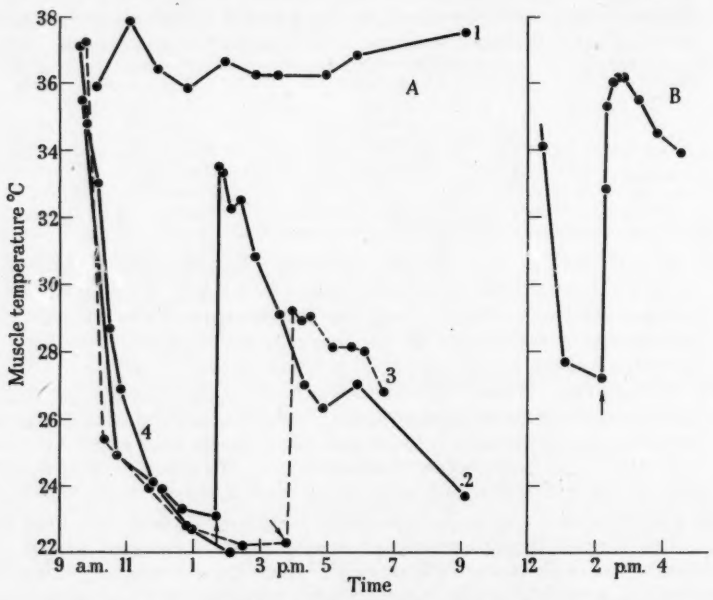


FIG. 1.—Temperature in the hind-limb muscle of the rat after various procedures.

A. Curve 1. Temperature in the left gastrocnemius muscle in a rat given a 3 min. period of ether anaesthesia 3 min. before the commencement of the recording. Environmental temperature 18°.

Curve 2. The effect of a 4 hr. period of bilateral hind-limb ischaemia on the temperature in the left gastrocnemius muscle. The tourniquets were applied under ether anaesthesia 4 min. before the commencement of the recording and removed at the arrow. The rat died overnight. Environmental temperature 18°.

Curve 3. The effect of a 6 hr. period of bilateral hind-limb ischaemia on the temperature in the left gastrocnemius muscle. The tourniquets were applied under ether anaesthesia 1 min. before the commencement of the recording and removed at the arrow. The rat died overnight. Environmental temperature 20°.

Curve 4. Temperature in the left gastrocnemius muscle of a rat killed immediately after the first observation. Environmental temperature 21°.

B. The effect of a 2 hr. period of bilateral hind-limb ischaemia on the temperature in the soleus muscle. The tourniquets were applied under ether anaesthesia 2 min. before the commencement of the recording and removed at the arrow. Environmental temperature 21°.

muscles was not as great as after 4 hr. ischaemia (Fig. 1A) and the maximum temperature was only 29·0° ± 0·8 in 10 rats. At an air temperature of 30° the rise in the temperature of the hind-limb muscle when the tourniquets were removed after 4 hr. was very slight (Fig. 2). In 5 experiments the average maximum temperature was 33·2° ± 0·5.

Although several factors determine muscle temperature its increase after ischaemia will be largely dependent on its blood flow so that this rise will be a measure of that flow. The secondary fall in temperature will depend on local and general factors, but it may be taken as evidence of a fall in the circulation through the limb. At ordinary environmental temperatures, the shorter the period of ischaemia the more complete is the restoration of the blood flow afterwards. The poorest return of the circulation was after a 4 hr. period of bilateral hind-limb ischaemia at an environmental temperature of 30°.

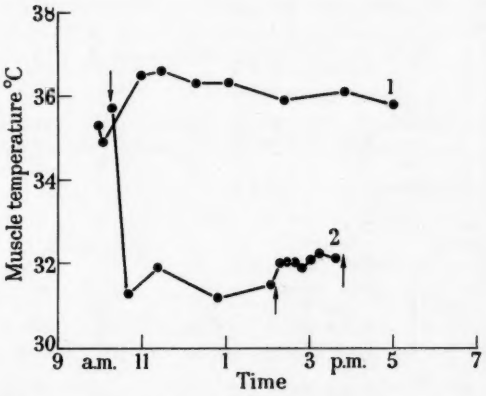


FIG. 2.—Temperature in the hind-limb muscle of the rat after various procedures at an environmental temperature of 30°.

Curve 1. The temperature in the soleus muscle in a rat given a 3 min. period of ether anaesthesia 4 min. before the commencement of the recording.

Curve 2. The effect of a 4 hr. period of bilateral hind-limb ischaemia on the temperature in the right gastrocnemius muscle. The tourniquets were applied under ether anaesthesia at the first arrow, 2 min. before the commencement of the recording. They were removed at the second arrow and the rat died at the third arrow.

Chemical changes during hind-limb ischaemia

In normal rats the concentrations of the compounds investigated remained constant over a 24 hr. period and were unaffected by cold-acclimatization. The control samples (Tables II, IV, VII) were taken between 2.30 and 3.30 p.m. Differences between the values for the fore- and hind-limb muscles may reflect differences between the muscles (Lawrie, 1953) but since they are most obvious in the inorganic P and PC fractions they are more probably due to the removal of the hind-limb sample after that from the fore-limb. The absence of AMP confirms the finding of Mommaerts (1955) and indicates that nucleotide breakdown did not occur during the preparation of the samples.

TABLE II.—*The Changes in the Acid-soluble Phosphate Fractions of the Hind-limb Muscle of the Rat during and after Bilateral Hind-limb Ischaemia and during Autolysis*

Duration of ischaemia or autolysis (hr.)	Time after removal of the tourniquets (hr.)	Number of rats	Muscle phosphate fractions μmoles total P per g. wet wt. (mean ± S.E.)						
			Total acid-sol. P	Inorganic P	PC–P	ATP	ADP	AMP	IMP
Normal rats—									
0	—	5	52·9±1·55	11·52±0·59	14·80±0·42	18·30±1·05	2·09±0·11	0	0
2	—	4	54·3±0·43	29·55±0·53	0·10±0·06	8·74±0·64	3·84±0·20	0·52±0·09	1·47±0·08
2	2	3	43·7±3·53	—	—	13·10±0·79	2·42±0·45	0·31±0·17	0·49
4	—	7	51·8±0·68	35·30±0·48	0·47±0·28	2·73±0·51	1·92±0·18	0	3·91±0·23
4	2–4	15	34·9±1·59	14·70±1·23	6·04±0·80	6·47±0·68	1·31±0·05	0	0·97±0·17
4	At death	9	32·3±2·50	18·60±1·17	3·30±1·07	4·36±1·10	1·42±0·15	0·17±0·08	0·82±0·32
4*	—	4	51·0±0·44	37·15±0·37	1·38±0·71	0·32±0·07	0·85±0·04	0	3·52±0·35
4*	0·5	8	38·8±1·55	29·40±1·16	0·70±0·40	0·34±0·09	0·77±0·10	0	2·35±0·24
6	—	3	52·8	37·20	0·10	0	1·60±0·31	0·44±0·05	5·33±0·32
6	0·5–1·25	6	36·9±0·37	28·20±0·65	6·65±0·30	0	0·68±0·04	0	2·60±0·22
Cold-acclimatized rats—									
0	—	2	53·8	9·75	14·30	18·40	2·22	0	0
4	—	3	49·9±1·77	29·40±0·76	1·90±0·55	2·12±0·48	1·65±0·21	0·22±0·04	4·19±6·19
4	2·5	3	23·6±3·20	10·52±0·37	3·70±0·15	4·64±0·75	1·20±0·41	0·11±0·04	0·19±0·08
Dead rats(autolysis)—									
2	—	3	56·9±1·15	29·90±1·10	0	13·25±0·45	5·10±1·35	0·30±0·02	1·01±0·11
4	—	3	55·5±0·87	42·00±1·63	0	1·16±0·35	1·87±0·27	0·39±0·05	5·12±0·36
6	—	3	57·3±0·60	48·00±0·87	0	0	0·82±0·08	0	6·09±0·36

The environmental temperature was 18–22° except for the groups indicated* where the experiments were carried out at an air temperature of 30°. The results of the statistical analysis of the values are given in the text where necessary.

During ischaemia (Tables II, IV) the total acid-soluble P concentration in the muscle was unaltered as was its water content. The most striking changes in the ischaemic muscle were in the ~ P fractions. PC was affected first and after 2 hr. had almost completely disappeared. This was followed by a reduction in the concentration of ATP, UTP and GTP so that after 4 hr. only small amounts of these compounds remained. The P liberated by these reactions was added to the inorganic P fraction.

Ischaemia also affected the other nucleotide fractions (Table II). The ADP concentration was first increased but later fell to approximately normal so that after 6 hr. it was the main adenine nucleotide component of the muscle. AMP was present in appreciable amounts after 2 and 6 but not after 4 hr. ischaemia. The IMP level in the ischaemic muscle rose steadily. Since the muscle was completely ischaemic, deamination of the accumulating AMP was to be expected (Macfarlane and Spooner, 1946). Nucleotide was not broken down beyond IMP and the nucleotide lost from the other fractions could be accounted for, within experimental error, as IMP (Table III). No evidence was found for the two unknown inosine compounds reported by Jordan and Gray (1955).

TABLE III.—*Comparison of the Disappearance of Adenine Nucleotide and the Accumulation of Inosine Monophosphate in Ischaemic and Autolytic Muscle*

Compound	Hind-limb muscle		
	Control	After 6 hr. ischaemia	After 6 hr. autolysis
	(μmoles per g. wet wt.)		
ATP	6·1	0	0
ADP	1·05	0·80	0·41
AMP	0	0·44	0
Total adenine nucleotide	7·15	1·24	0·41
Loss of „ „	0	5·91	6·74
IMP	0	5·23	6·09

These values have been taken from Table II and have been converted from μmoles P to μmoles compound per g. wet wt.

DPN was more resistant to the effects of anoxia than the other compounds (Table IV). At an air temperature of 18–22° the DPN concentration was not reduced until after 6 hr. ischaemia when most of the loss could be accounted for as DPNH. This may be related to the diminished rate of glycolysis at that time (Table V).

Cold-acclimatization did not affect the changes in the muscle during ischaemia. The changes in normal rats were accelerated by raising the environmental temperature to 30° (Tables II and IV).

The changes during autolysis were also studied. The rats used were killed by an overdose of Na pentobarbitone so that they died without struggling and the initial fall in temperature was similar to that in the tourniquet experiments (Fig. 1A). The pattern of the changes in the acid-soluble phosphate fractions of the muscle during the first 6 hr. after death was similar to that during ischaemia (Table II). These results are in fair agreement with those of Bendall (1951) and Bate-Smith and Bendall (1956) in the rabbit when allowance is made for the

TABLE IV.—*Changes in the Coenzyme I, GTP, UTP and Water Contents of Rat Hind-limb Muscle during and after Bilateral Hind-limb Ischaemia and during Autolysis*

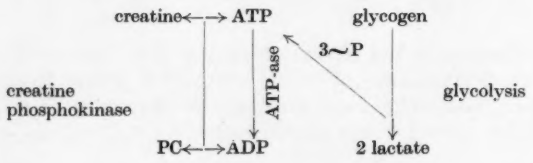
Duration of ischaemia (hr.)	Time after removal of the tourniquets (hr.)	Number of rats	μmoles total P per g, wet wt. (mean ± S.E.) DPN	DPNH	GTP+UTP	% Water
0	—	5	1·02±0·13	0	0·44±0·02	75·8±0·18
2	—	4	1·01±0·04	0	—	—
2	2	3	0·83±0·16	0	—	—
4	—	7	1·01±0·11	0	0·16±0·07	76·5±0·44
4	2–4	15	0·52±0·05	—	0·40±0·05	80·9±0·93
4	At death	9	0·39±0·06	—	—	—
4*	—	4	0·32±0·03	0·24±0·04	—	—
4*	0·5	8	0·23±0·04	0	—	82·5±2·7
6	—	3	0·51±0·05	0·38	—	76·6±0·30
6	0·5–1·25	6	0·26±0·02	0·25±0·03	—	81·7±0·23
Duration of autolysis (hr.)						
2	—	3	0·67±0·13	0	—	—
4	—	3	0·57±0·11	0·17±0·06	—	—
6	—	3	0·62±0·08	0·22±0·03	—	—

The environmental temperature was 18°–22° except for the groups indicated* where the experiments were carried out at an air temperature of 30°.

initial total glycogen concentration which in our rats was 32·3 ± 2·3 μmoles glucose per g. wet wt. muscle (Stoner, unpublished results). The fall in the concentration of adenine nucleotide could again be accounted for as IMP (Table III) precluding the formation of other purine derivatives (Kerr and Seraidarian, 1945).

The myokinase reaction and " ATP turnover " in ischaemic muscle

From current biochemical evidence the initial reactions occurring in ischaemic muscle could be expressed as follows :



The subsequent degradation of ADP would be through the myokinase reaction

$$2ADP \rightleftharpoons ATP + AMP$$

and the AMP formed would be deaminated by adenylate deaminase

$$AMP \longrightarrow IMP + NH_3$$

The findings recorded in Table II are in agreement with this scheme. Accepting that dephosphorylation of ATP to ADP is the primary reaction, the disappearance

of PC before ATP and before the major accumulation of IMP would be expected since the available evidence (Eggleston and Hems, 1952 ; Noda, Kuby and Lardy, 1954) suggests that while the equilibrium in both the creatine phosphokinase and myokinase reactions favours ATP formation, the former is faster and the full force of the latter will not be felt until the PC stores are exhausted. This is probably why the major accumulation of IMP is in the later part of the ischaemic period when conditions will be ideal for the action of adenylate deaminase (Nikiforuk and Colowick, 1956). Since, *in vitro*, the adenylate deaminase reaction proceeds to completion as does the myokinase reaction in the presence of adenylate deaminase (Colowick and Kalckar, 1943) IMP and not AMP should be the final product and this is borne out by the results.

The data provide quantitative evidence for the existence of the myokinase reaction in ischaemic muscle. In certain instances a reaction constant for the myokinase reaction can be calculated according to the formula

$$k = \frac{[\text{ATP}].\ [\text{AMP}].}{[\text{ADP}]^2}$$

The values of k obtained after 2 hr. periods of ischaemia (0·41) or autolysis (0·41) and at death after 4 hr. ischaemia (0·49) agree very well with that of 0·44 obtained by Eggleston and Hems (1952) and Bowen and Kerwin (1956) in an *in vitro* system. The low value of k after 4 hr. autolysis (0·16) and the persistence of ADP after 4 and 6 hr. ischaemia suggests some interference with the myokinase reaction by prolonged anoxia.

During ischaemia the ATP concentration in the muscle will be maintained by $\sim$P from three sources which, in order of importance, are glycogen breakdown, PC and the myokinase reaction. In parallel experiments (Stoner, unpublished results) the average loss of muscle glycogen during the first 2 hr. period of ischaemia was 7·67 μmoles, during the second 2 hr. period 16·30 μmoles, and during the third 2·30 μmoles glucose per g. wet wt. from which the contribution from glycolysis can be calculated assuming that each μmole of glycogen glucose converted to lactate will yield 3 μatoms $\sim$P. The further study of these muscle extracts referred to in the introduction showed that there is no interference with glycolysis in ischaemic muscle. The μatoms $\sim$P from the myokinase reaction will be equivalent to $\frac{1}{2}$ sum (in μmoles) of the AMP and IMP formed. A " turnover rate " for the terminal $\sim$P of ATP in ischaemic muscle can therefore be calculated as done by Bate-Smith and Bendall (1949) for autolytic muscle with the addition that it is now possible to allow for the small contribution from the myokinase reaction. Allowing for the temperature difference, the results for the first 4 hr. of the ischaemic period (Table V) are of the same order as the value of 0·81 μatoms $\sim$P per min. per g. given by Kalckar, Dehlinger and Mehler (1944) for intact living rabbit muscle. The Q_{10} for most of the reactions involved is between 1·61 and 1·65 (Bendall, 1951). In autolytic muscle the rate of ATP " turnover " depends on the species, temperature, initial glycogen level and time after death (Bendall, 1951 ; Bate-Smith and Bendall, 1956). When time is the only variable the rate first falls, rises again and then drops steeply as extensibility is lost completely. Because of this it is difficult to compare our results directly with those of Bate-Smith and Bendall but they are of the same order. The low "turnover rate" during the third 2 hr. period of ischaemia is presumably due to exhaustion of material for the anaerobic synthesis of $\sim$P.

TABLE V.—*"Turnover Rate" of the Terminal ~ P in ATP in Ischaemic Rat Muscle*

	Period of ischaemia		
	0–2 hr.	2–4 hr.	4–6 hr.
	(μatom terminal ~ P per g. wet wt.)		
Fall in ATP level	3·19	2·00	0·91
~ P from PC	14·65	0	0·37
~ P from glycolysis	23·00	49·00	6·90
~ P from myokinase reaction	1·0	1·96	0·93
Total turnover	41·84	52·96	9·11
"Turnover Rate" μatom ~ P/min./g. wet wt.	0·35	0·44	0·076

The chemical changes in the hind-limb after ischaemia

There was little loss of acid-soluble phosphate (Table II) or total nucleotide (Table VI) and the ATP concentration showed good recovery 2 hr. after a 2 hr. period of ischaemia. The amount of ADP present was normal and although AMP and IMP were still found their concentrations were less than at the end of the ischaemic period.

TABLE VI.—*Changes in the Total Nucleotide Content of the Damaged Muscle after Removal of the Tourniquets*

Period of ischaemia (hr.)	Time after removal of tourniquets (hr.)	Total nucleotide content of hind-limb muscle μmoles per g. wet wt.	Per cent loss
0	0	7·15	—
2	2	6·38	10·8
4	2–4	3·75	47·5
4†	2–4	2·45	65·7
4	At death	3·15	55·8
4*	0·5*	2·85	60·2
6	0·5–1·25	2·94	59·0

Values calculated from data in Table II.
* = air temperature 30°; for others 18–22°.
† = cold-acclimatized rats.

Between 2 and 4 hr. ischaemia at an environmental temperature of 18–22° a critical point is reached which determines whether the total nucleotide concentration in the muscle will be maintained or fall when the tourniquets are removed (Table VI). Ischaemia of 4 hr. or longer is followed by a progressive fall in the total nucleotide content chiefly during the first 4 hr. after removal of the tourniquets. At the same time there is a difference in the nucleotide pattern in the muscle depending on whether the ischaemia has lasted 4 or 6 hr.

The resynthesis of energy-rich phosphate

When muscle has been ischaemic for a certain period it is thought to be unable to synthesize ~ P when the blood flow through it is restored. The duration of the ischaemia necessary to produce this effect was put at 3 hr. by Bollman and Flock (1944) and Macfarlane and Spooner (1946). From estimations on the muscle it is not possible to distinguish between the inability to synthesize a constituent and the failure to store it after synthesis. Muscle permeability is altered after ischaemia and large molecules such as myohaemoglobin pass readily into the returning blood and lymph (Montagnani and Simeone, 1953). In the present

experiments the duration of ischaemia, at an environmental temperature of 18–22 ° necessary to prevent an increase in the muscle ~ P content after restoration of the blood flow, was between 4 and 6 hr.—longer than that necesssary for the effect on total nucleotide content (Table VI).

When muscle which had been ischaemic for 4 hr. was examined 2–4 hr. later a net gain of ~ P in the form of PC, ATP, GTP and UTP was found to have occurred ($P < 0{\cdot}01$). It will in fact be slightly greater than shown in Table II since those values have not been corrected for the increased water content of the tissue. Part of the increased ATP content is due to the phosphorylation of ADP but there also seems to be an increase of about 0·9 μmole per g. wet wt. in the adenine nucleotide content of the muscle. This is the difference between the mean adenine nucleotide content, in μmoles per g. wet wt., of hind-limb muscle at the end of the ischaemic period and 2–4 hr. later, *i.e.*, $1{\cdot}91 \pm 0{\cdot}20$ (7 rats) and $2{\cdot}81 \pm 0{\cdot}24$ (15 rats). This difference is only just statistically significant at the $P = 0{\cdot}05$ level. Additional evidence would be required before re-amination of the IMP in the muscle could be postulated with any confidence.

Qualitative evidence for resynthesis of ATP in muscle which had been ischaemic for 4 hr. was obtained when ^{32}P was injected intramuscularly into the fore-limb a few minutes after removal of the tourniquets and the hind-limb muscle removed 2–2·5 hr. later. Perfusion of the muscle before excision reduced the chances of contamination with highly active inorganic P and excluded the possibility of the ATP in the extracts being derived from erythrocytes. The ATP spots on the chromatograms from all the 6 rats studied in this way showed significant radioactivity. While the absence of activity in these areas would have been definite evidence against resynthesis, its presence may not be unequivocal evidence of resynthesis since Ernster, Ljunggren and Lindberg (1954) and Boyer, Falcone and Harrison (1954) have shown the possibility of incorporating ^{32}P into ATP by exchange reactions.

The recovery of the muscle ATP content after 4 hr. ischaemia varied considerably. This could be due to differences in the volume of blood flowing through the limbs after removal of the tourniquets. Chemical and temperature observations on the hind-limb muscles were available for 8 rats. In general the amount of ~ P in the PC and terminal P of the ATP in the muscles 2–3 hr. after removal of the tourniquets was correlated with the rise in the muscle temperature. The higher and the better sustained that rise the more ~ P was found in the muscle. These results support the view that the better the circulation through the damaged muscle the better the accumulation of ATP. This is also borne out by the findings after 6 hr. ischaemia and after 4 hr. ischaemia at an environmental temperature of 30°. In the cold-acclimatized rats where the restoration of ATP was about the same as in unacclimatized rats (Table II) the changes in muscle temperature were also similar.

When hind-limb muscle was examined 0·5–1·25 hr. after a 6 hr. period of ischaemia (Table II) the loss of inorganic P was not great. Despite the continued absence of ATP, there was an increase in PC content in some cases although this rise was very much less than after 4 hr. ischaemia. The muscle at this time was not only devoid of ATP but the ADP ($P < 0{\cdot}01$) and AMP concentrations were lower than when the tourniquets were released. For 0·5 hr. after a 4 hr. period of hind-limb ischaemia at an environmental temperature of 30° the only significant changes were the reductions in the total acid-soluble P ($P < 0{\cdot}001$), inorganic P

($P < 0{\cdot}01$) and IMP ($P < 0{\cdot}05$) levels in the hind-limb muscle (Table II). In neither case was there any evidence for the resynthesis of ATP, indeed in the former the breakdown of adenine nucleotide continued after the tourniquets were removed.

Loss of nucleotide from muscle after ischaemia

When the circulation was restored to muscle which had been ischaemic for 4 hr. or longer there was a progressive decline in its total nucleotide content, mostly within a few hours of removing the tourniquets. This was largely accounted for by the fall in its IMP content. This could be explained by its passage into the blood or lymph or by participation in further chemical reactions.

Uptake of ^{15}N into the 6-amino group of adenine nucleotide in normal rat muscle has been shown by Kalckar and Rittenberg (1947) but the re-amination of IMP requires DPN and a source of $\sim$ P in addition to aspartic acid as the N source (Abrams and Bentley, 1955*a*, *b* ; Newton and Perry, 1957) so that conditions in the damaged muscle might not be suitable. The results shown in Table II do not favour re-amination particularly after 6 hr. ischaemia or 4 hr. ischaemia at 30° air temperature. Many nucleotide transphosphorylations of the type RTP + R′MP $\rightleftharpoons$ RDP + R′DP have been described but IMP does not appear to take part in them (Krebs and Hems, 1955 ; Gibson, Ayengar and Sanadi, 1956). IMP can act as phosphate donor in nucleoside phosphotransferase reactions but the activity of muscle in this respect is low (Brawerman and Chargaff, 1955). Since the total nucleotide concentration of the muscle was reduced (Table VI) such reactions, which would tend to maintain that level, are also unlikely.

Direct attempts to demonstrate the release of IMP into the blood stream were unsuccessful. Plasma from samples of aortic blood taken shortly after release of the tourniquets did not contain any detectable nucleotide. In 4 rats blood obtained from the inferior vena cava, after occluding it below the entry of the renal veins and with the needle of the syringe pointing towards the hind limbs so as to collect their venous outflow, was also tested. The sensitivity of the method is 0·1 μmole per ml. and IMP was only detected in one plasma sample. No other nucleotides were seen in these samples.

Judging by the state of the muscle at death IMP disappears slowly during the survival period. In the hind-limb muscle of rats dying after a 4 hr. period of ischaemia there was also a rise ($P < 0{\cdot}05$) in the inorganic P concentration and the reappearance of AMP. At the same time there was a small reduction in the amounts of PC and ATP although these changes were not statistically significant. This suggests a terminal recurrence of ischaemia and the temperature and appearance of the limb at that time were in accordance with this. Nevertheless the mean concentrations of PC ($P < 0{\cdot}05$) and ATP in the muscle at this time were still higher than at the end of the 4 hr. period of ischaemia.

The chemical changes in the uninjured muscle

There were no changes in the acid-soluble P fractions of the fore-limb muscle during ischaemia of the hind-limbs (Table VII). The state of these fractions 2–4 hr. after removal of the tourniquets and at death after 4 hr. bilateral hind-limb ischaemia at an air temperature of 18–22° (Table VII) confirms and amplifies our previous findings (Stoner and Threlfall, 1954). In all the samples shown in Table VII the DPN level was within normal limits and no DPNH was found. The

TABLE VII.—*Changes in the Acid-soluble Phosphate Fractions of the Fore-limb Muscle of the Rat during and after Bilateral Hind-limb Ischaemia*

Duration of ischaemia (hr.)	Time after removal of the tourniquets (hr.)	Number of rats	Muscle phosphate fractions μmoles total P per g. wet wt. (mean ± S.E.)					
			Total acid-sol. P	Inorganic P	PC–P	ATP	ADP	AMP	IMP
Normal rats—									
0	—	5	51·4±1·47	9·30±0·53	16·10±0·15	18·70±0·40	2·26±0·10	0	0
4	—	7	52·6±0·93	11·00±0·18	16·80±0·49	18·10±0·54	2·03±0·17	0	0
4	2–4	11	53·4±3·06	11·86±0·89	14·34±0·77	17·24±0·75	1·99±0·14	0	0
4	At death	9	55·2±1·62	22·90±1·19	6·90±1·11	14·97±1·54	2·53±0·13	0	0·90±0·45
4*	0·5	8	56·0±1·30	13·60±1·69	10·10±1·74	15·40±0·98	2·34±0·23	0·07±0·05	0·15±0·09
Cold-acclimatized rats—									
0	—	2	54·2	7·95	16·40	17·30	2·52	0	0
4	—	3	54·8±3·15	6·36±1·32	17·08±0·81	17·40±1·08	2·22±0·67	0	0·33±0·21
4	2·5	3	51·9±0·75	6·43±0·40	10·70±0·54	16·30±0·84	1·71±0·13	0	0

The environmental temperature was 18–22° except for the group indicated* where the experiments were carried out at an air temperature of 30°. The results of the statistical analysis of the values are given in the text where necessary.

water content of these samples was also normal except in those taken 0·5 hr. after 4 hr. ischaemia at an air temperature of 30° when it was reduced to 75·2 ± 0·20 per cent ($P < 0·01$). At death the PC ($P < 0·001$) and ATP ($P < 0·05$) contents of the muscle were reduced with a corresponding increase in the inorganic P ($P < 0·001$) and IMP levels. Since no phosphate had left the muscle these changes were probably the result of agonal local ischaemia.

At an environmental temperature of 30° more pronounced changes (Table VII) were found in the fore-limb muscle 30 min. after removal of the tourniquets than previously (Stoner and Threlfall, 1954). Although the muscle contained large amounts of ~ P there had been some breakdown with an increase in inorganic P and the appearance of AMP and IMP. Since the total acid-soluble P concentration did not fall these changes were probably due to local anoxia. The differences between the two sets of results are partly due to improvements in chemical technique but the main difficulty arises from the variability in the survival time. At this environmental temperature the rats die very suddenly with little external warning so that it is impossible to be sure that all the members of the group are in the same condition.

DISCUSSION

By applying recent analytical developments it has been possible to obtain a fairly complete picture of the changes in the energy-rich phosphates and nucleotides of muscle during and after ischaemia.

In ischaemic muscle the first change is the dephosphorylation of ATP to ADP but the ATP level in anoxic muscle falls relatively slowly since every means is taken to preserve it, by the rephosphorylation of ADP in the creatine phosphokinase reaction, by the production of ~ P in the breakdown of glycogen stores and by the breakdown of ADP itself in the myokinase reaction. When the stores of PC and glycogen are depleted ATP eventually disappears and the excess ADP is broken down through the myokinase reaction to give AMP which is deaminated to IMP. The persistence of appreciable amounts of ADP in muscle even after long periods of ischaemia may be due to the effect of prolonged anoxia on myokinase. It indicates that ADP cannot be dephosphorylated or deaminated directly in muscle *in vivo* although Webster (1953) has reported that it can be deaminated by isolated myofibrils. In the intact muscle the myokinase reaction seems the only pathway between ADP and AMP. The final product is IMP which accumulates in the ischaemic muscle. Similar changes occur in UTP and GTP and if the conditions are sufficiently severe DPN is converted to DPNH and the total coenzyme I concentration falls.

These changes are similar to those which occur during autolysis and are influenced by the same factors, namely the temperature of the muscle, the duration of the anoxia and the initial concentration of possible sources of ~ P (PC and glycogen). The first two factors are the most important in practice. The higher the muscle temperature and the longer the period of ischaemia the more marked the changes will be.

This scheme applies to skeletal muscle. Anoxia of other tissues also reduces their nucleotide and PC contents but the precise steps involved may be different (Kerr and Seraidarian, 1945). In autolysing brain, for instance, AMP is formed and disappears but IMP does not accumulate.

Since the work of Bollman and Flock (1944) it has been thought that the

duration of the ischaemia determined whether or not resynthesis of ~ P occurred. Their results showed that if ischaemia lasted more than 3 hr. the ATP level did not rise afterwards. Most workers (Macfarlane and Spooner, 1946; LePage, 1946; Kovách *et al.*, 1952; Jordan and Gray, 1955) have supported this view but Harman and Gwinn (1949) reported the re-accumulation of ~ P 24 hr. after 4 hr. unilateral hind-limb ischaemia. The present experiments show resynthesis of ATP after periods of bilateral ischaemia up to and including 4 hr. at an environmental temperature of 18–22°. A longer period or a higher environmental temperature was required to prevent the re-accumulation of ~ P.

Although the muscles of the hind-limb are in effective communication with the general circulation when the tourniquets are removed the complexity of the changes in their blood flow has not been generally realized. As Harman (1948) and Harman and Gwinn (1949) have shown, in many cases there is a continuing relative ischaemia after removal of the tourniquets. If the changes in muscle temperature can be taken as a valid indication of changes in blood flow then there is a good correlation between the resynthesis of ~ P and the degree to which the circulation is restored. This seems more significant since it covers a much wider range of experimental situations and might explain some of the anomalies. The duration of anoxia is only one of the factors determining the blood flow through the damaged muscle. In the results of Harman and Gwinn (1949), 4 hr. unilateral hind-limb ischaemia rarely produces fatal shock and will be followed by a much better return of the circulation than when both hind-limbs are injured. It seems to us that if ~ P fails to be resynthesized after ischaemia it is because the blood flow through the muscle is inadequate and not because of a primary irreversible change in the muscle cells. If the blood flow through the muscle after ischaemia reaches a critical level ~ P synthesis will occur. A further problem is whether this resynthesis is evenly distributed in the muscle or whether it is concentrated in those fibres whose anatomical integrity is best preserved. The histological observations of Harman and Gwinn (1949) suggest the latter. This might seem at variance with the emphasis we have placed on the rôle of the circulation but since the integrity of the cell is also dependent on its blood supply it may be another way of expressing the same idea.

It is generally agreed that inorganic P is washed out of the ischaemic muscle when the circulation is restored. Our results show that under these conditions the muscle also loses nucleotide. The extent of the loss seems more closely related to the duration of the anoxia (Table VI) than to the changes in blood flow afterwards and is probably determined by the damage to the permeability of the muscle during the anoxia. The degree to which restoration of the circulation relieves the anoxia will play some part in that it will tend to stop further damage and reverse the changes which have occurred but, under our conditions, this part seems a minor one.

The main question is the form in which the nucleotide leaves the muscle. Bollman and Flock (1944) and Macfarlane and Spooner (1946) argued that nucleotide does not leave the muscle as ATP since this is virtually absent after 4 hr. ischaemia and is not resynthesized. As previously pointed out (Green and Stoner, 1950*a*, *b*, *c*) this argument fails because their experiments only measured the ability of the muscle to store ATP. From the results of Bielschowsky and Green (1945) it was possible that the damaged muscle was resynthesizing ATP which then passed out into the extracellular space. Although this idea now seems unlikely

it cannot be completely disproved, especially as the resynthesis of ATP after 4 hr. bilateral hind-limb ischaemia has now been shown *in vivo*.

The main loss is from the IMP fraction and the simplest explanation is that it passes out of the muscle as such. Where resynthesis of ATP occurred more evidence would be needed before concluding that it represented more than the phosphorylation of available adenine nucleotide. Where no resynthesis occurred the changes indicate the continuing degradation of the adenine nucleotides. The results in Table II would fit the hypothesis that most of the IMP formed during anoxia is removed by the circulation, but they do not prove it.

The absence of adenyl compounds from the plasma after injury confirms our previous findings in the rat using biological assay methods (Green, Stoner and Bielschowsky, 1949). Of the nucleotide breakdown products inorganic P and pentose accumulate in the plasma of the rat after limb ischaemia (McShan, Potter, Goldman, Shipley and Meyer, 1945 ; Green *et al.*, 1949). However, this throws little light on the form in which these compounds enter the circulation. Extra-cellular nucleotide is rapidly broken down in the rat so that after the intramuscular injection of fatal doses of ATP no adenyl compounds appear in the plasma, but only pentose (Green *et al.*, 1949 ; Green and Stoner, 1950*a*). The deaminase activity of the blood is also greatly increased by the application of tourniquets (Drury A. N., personal communication). Since IMP does not accumulate in the plasma, although when added to rat blood *in vitro* it does not rapidly disappear from the plasma, failure to detect it in the venous effluent from the limb was to be expected. The amount of IMP which disappears from the muscle is such that if it slowly passed from the muscle to the circulation intact its level in the plasma need not reach the threshold of the method. The absence of significant amounts of pharmacologically active nucleotides and their derivatives from the blood of the rat after limb ischaemia helps to supply a negative answer to the question—do the adenine nucleotides play any part in the causation of the general responses to injury? (Green and Stoner, 1950*b*). There is no evidence that these compounds act like an "exogenous" toxin in the general response to limb ischaemia in the rat. Further work should now be done on other species such as the rabbit where increases in the "adenosine equivalent" of the blood have been reported after limb ischaemia (Billings and Maegraith, 1937 ; Stoner and Green, 1944) and after the intramuscular injection of ATP (Stoner and Green, 1949).

The catabolism of the $\sim$ P compounds in the injured muscle has been causally related to shock in other ways. Macfarlane and Spooner (1946) suggested that since the permeability of the muscle was related to its $\sim$ P concentration the greater the fall in this level during ischaemia and the less the resynthesis afterwards, the greater would be the local loss of fluid and the greater the degree of shock. Bollman and Flock (1944) showed that while 2 hr. bilateral hind-limb ischaemia was never fatal, it was if combined with electrical stimulation of the muscles so as to produce the chemical picture seen in the muscles after 4 hr. ischaemia. In our experiments on normal rats comparison of the mortality rate and survival time with the duration of ischaemia and the changes in muscle chemistry seem to support these ideas. But the effect of 4 hr. bilateral hind-limb ischaemia on the muscle of the cold-acclimatized rats was the same as in normal rats, yet the mortality rate was much less. While the changes in the damaged muscle initiate the response they do not necessarily determine the outcome.

The idea that the reduced $\sim$ P concentration in the injured areas could initiate

and even decide the result of the general response to injury was used by Potter and his colleagues (McShan *et al.*, 1945; LePage, 1946) in their development of the "energy depletion" hypothesis of shock. The present results support our previous conclusion that this hypothesis can only be applied when death takes place under certain conditions (Stoner and Threlfall, 1954).

In conclusion it may be said that while a more complete description of the effect of ischaemic injury on muscle has helped to resolve some of the problems concerning this form of injury, many still remain.

SUMMARY

The effect of hind-limb ischaemia on the inorganic phosphate, phosphocreatine, adenine nucleotide, inosine monophosphate, guanosine and uridine triphosphate and coenzyme I concentrations of the fore- and hind-limbs has been observed in the rat. The changes during ischaemia were compared with those found during autolysis. An attempt has also been made to correlate the changes in the phosphate compounds with those in the circulation through the limbs and with the general response of the animals. The experiments were carried out on normal rats at two environmental temperatures (18–22° and 30°) and on cold-acclimatized rats at 18–22°.

During complete ischaemia of the hind-limbs the changes in the muscle were the same as during autolysis. The energy-rich phosphates were broken down and inosine monophosphate accumulated as the end product of the adenine nucleotide series. The degree of breakdown depended on the duration of the ischaemia and the temperature of the muscle. Evidence for the myokinase reaction was found in the ischaemic muscle and its "adenosine triphosphate turnover" was calculated.

After ischaemia the restoration of the energy-rich phosphate levels in the muscle appears to depend on the degree to which the circulation is restored rather than simply on the duration of ischaemia.

In addition to inorganic phosphate, nucleotide disappeared from the damaged muscle if it had been ischaemic for more than 2 hr. The fate of this nucleotide has not been established. The evidence would accord with its release from the muscle as inosine monophosphate.

The previous conclusion that limb ischaemia does not cause any important change in the energy-rich phosphate concentration of the muscle outside the damaged area was confirmed.

Cold-acclimatization increased the resistance of the rats to limb ischaemia although the chemical changes in the muscle were unaltered.

The bearing of these findings on current views on the effect of ischaemic injury on metabolism is discussed.

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FURTHER EVIDENCE OF THE ANTILEPTOSPIRAL EFFECT OF MILK: ELECTRON MICROSCOPIC STUDIES*

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WE have reported previously (Kirschner and Maguire, 1955) that leptospira rapidly died in undiluted milk. This natural antileptospiral effect was found in milk from various donors (human beings, cows and goats) and in human colostrum. The studies were made by investigations under the dark-ground microscope and by animal experiments. Rapid lysis was observed of both virulent and non-virulent strains of the three serological types tested (*Leptospira icterohaemorrhagiae*, *L. pomona*, *L. hyos*).

Its physical properties and the rapid lysis caused by it suggested that the agent might be lysozyme or lactenin. The mode of action was similar, but not identical with that of either. The antileptospiral principle was mainly associated with casein. It was concluded that the presence of this antileptospiral agent in cows' milk explained the absence of milk-borne infections in the many countries in which bovine leptospirosis is widely spread.

The studies have been continued with the use of the electron microscope in order to check the disintegration of leptospira at various intervals after mixture with milk, and to compare the lytic effect exerted by immune sera and 1 per cent sodium desoxycholate. The results are presented in this paper.

MATERIALS AND METHODS

Samples of fresh milk were collected for our purposes from the Milk Treatment Station immediately on arrival from farms. The fat was removed by repeated centrifugation and 1 ml. milk mixed with 0·1 ml. of well grown culture (3–4 days old) in modified Schüffner's medium and this was maintained at room temperature (17–19°). Three serotypes of leptospira (*L. icterohaemorrhagiae*, *L. pomona* and *L. hyos*) were used in these investigations. The number of organisms added to 1 ml. of milk was 2×10^6–2×10^7. All investigations were done at room temperature in order to approximate as nearly as possible to the environmental conditions on dairy farms.

As controls the same quantities of cultures were added to sterile tap water (pH 6·9–7·0).

Rabbit immune sera used in these investigations (agglutination-lysis titre 10,000) were diluted 1/10, 1/30, 1/100.

At intervals of 45 min., 12 hr. and 40 hr. the mixtures were centrifuged at 15,000 r.p.m. for 15 min. Both the supernatant and the deposit were examined under the dark-ground microscope. The supernatant was removed and the deposit used for further investigations. The deposit was washed six times with glass-distilled water. After the last washing the supernatant was discarded and the deposit re-suspended in 0·25 ml. distilled water. An

* Based on a communication to the Australian and New Zealand Association for the Advancement of Science, Dunedin, January, 1957.

equal quantity (0·25 ml.) of 0·1 per cent osmic acid was added for fixation for 10 min. The mixtures were again centrifuged, the supernatant discarded and the sediment re-suspended in 0·25 ml. distilled water. These samples were used for electron microscopy.

Preparation for electron microscopy

Small drops of the specimen were mounted on collodion film-covered grids, dried by a current of warm air, shadow-cast with uranium metal and examined with a Metropolitan-Vickers electron microscope EM2/1 installed at the Dominion Physical Laboratory in Lower Hutt, N.Z.

In some cases the collodion films were covered with an evaporated layer of carbon before applying the specimen material and the collodion removed after shadowing by immersing the grid in amyl acetate for 20 min. This process leaves the specimen material supported only by a film of carbon. Specimens so treated are much more stable in the electron beam.

The specimens were examined and micrographs taken at an initial magnification of 5000 × with subsequent photographic enlargement (to 9,000 × and 30,000 ×).

RESULTS

The electron micrograph (Fig. 1) of leptospira suspended in tap water shows the structure of leptospira as described by Swain (1955), Czekalowsky and Eaves (1955) and Mölbert (1955), consisting of a protoplasm cylinder and a fibril which is wound around it. This served as a control for the following micrographs.

The effect of milk on leptospira was first studied after an interval of 45 min. The progress of dissolution of the structure of the organisms is clearly seen in Fig. 2. Besides normal leptospira there were many organisms which showed only the resistant axial filament joining a mass of protoplasm.

After 3 hr. the number of leptospira was greatly reduced compared with the controls maintained in tap water. There were still next to damaged organisms some which appeared normal.

After 12 and 40 hr. many damaged leptospira were to be seen (Fig. 3) while in the controls (in tap water) the bulk of the organisms appeared normal (Fig. 3a).

Pasteurized milk (heated for 30 min. at 60°) had the same effect as fresh milk. The effects of milk were the same on all three serotypes of leptospira.

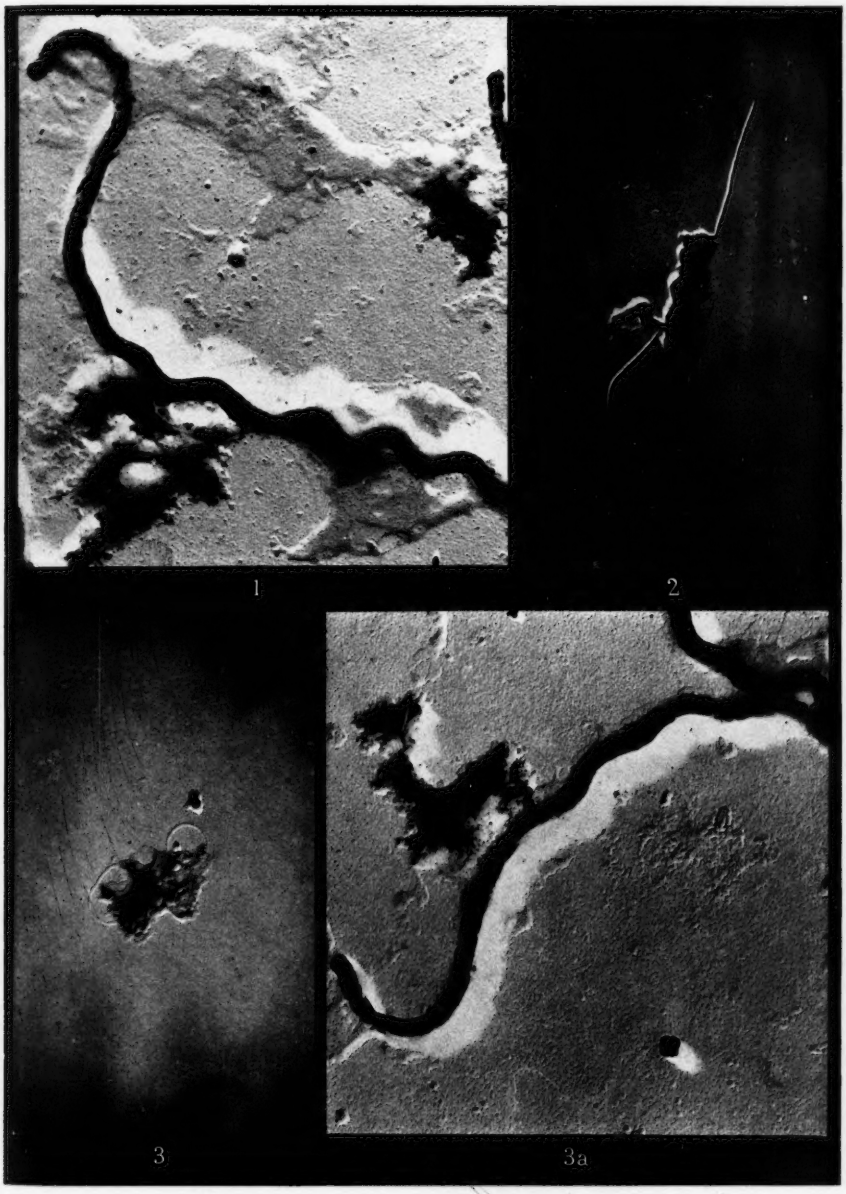
The lytic effect of immune antibodies on living leptospira was quite different. After an interval of 45 min. the organisms were tightly packed together in large clumps (lysis balls) or small spheric granules with complete loss of their structure. However some of the leptospira still showed normal appearance. After 12 and 40 hr. they had disappeared in the dilutions 1/10 to 1/100.

As control of our microscopic investigations to all tubes (content 1 ml.) with final dilutions 1/10, 1/30, 1/100, 1/300, 1/1000, 1/3000, 1/10,000, 2 ml. Schüffner medium was added and they were incubated at 30°. After 1–2 weeks there was

EXPLANATION OF PLATES

The serotype of leptospira in all the following electron micrographs is *L. pomona*.

FIG. 1.—Suspended in tap (rain) water. × 30,000.
FIG. 2.—Added to milk: after 45 min. × 9,000.
FIG. 3.—Added to milk: after 12 hr. × 9,000.
FIG. 3a.—Added to tap water: after 40 hr. × 30,000.
FIG. 4.—Agglutination of formolized leptospira. × 9,000.
FIG. 5.—Effect of 1 per cent sodium desoxycholate (30 min. after mixing). × 9,000.
FIG. 6.—Lysis by immune serum. × 9,000.
FIG. 7.—Cystic forms. × 9,000.



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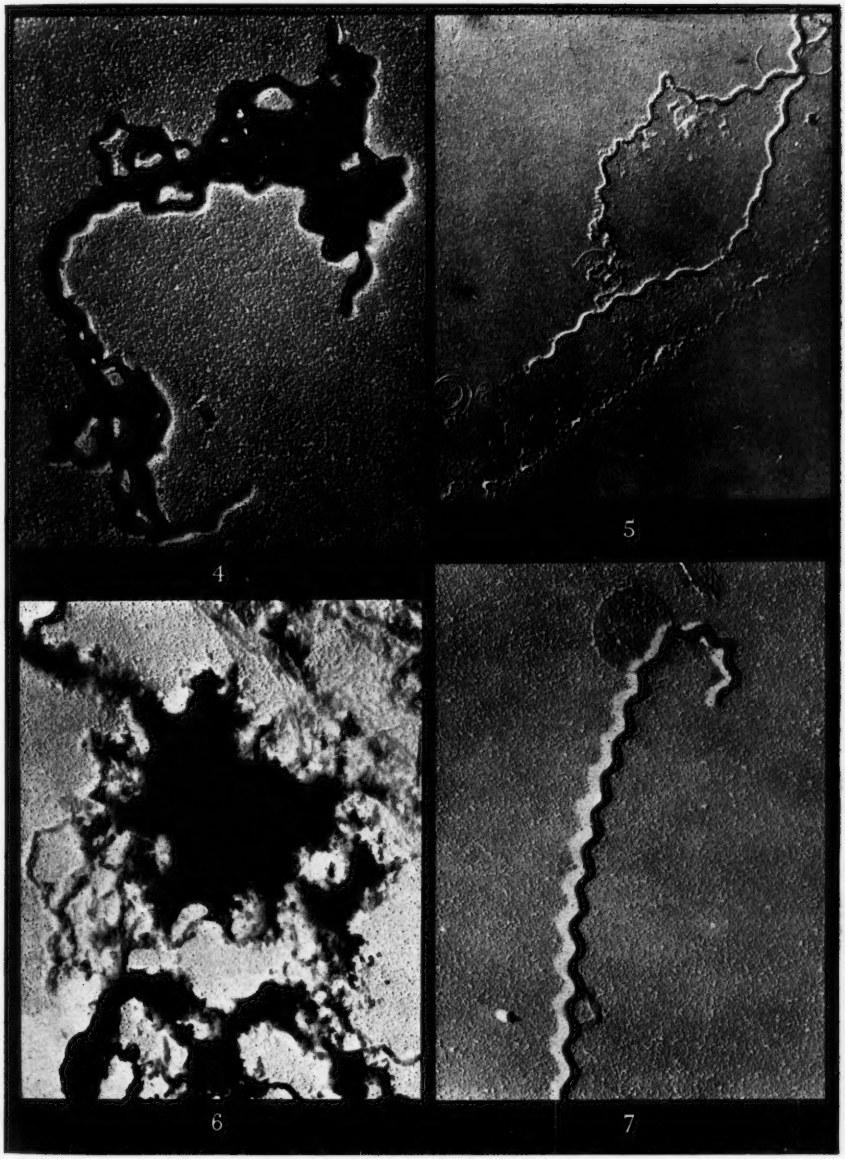

Kirschner, Maguire and Bertaud.

growth only in dilutions higher than 1/300. There was no difference in the results found in the sera examined heated 30 min. at 56° and unheated. This confirmed the very early findings of Schüffner and other Dutch workers (Wolff, 1924, 1954; Ruys, 1934; Ruys, Minkenhof and Wolff, 1948) that lysis of these organisms is not influenced by the presence of complement. The small granules and spheric shining masses, without any structure as a result of the lysis, remained unchanged throughout the whole time of observation. The number of leptospira not lysed in the dilution 1/300 was too small to initiate growth.

The known resistance of formolized leptospira to lytic antibodies is shown in Fig. 4. The agglutinated leptospira retained their forms.

The strong bacteriolytic effect of sodium desoxycholate on various organisms applies to leptospira as well. This is seen in Fig. 5, taken 30 min. after mixing 1 per cent sodium desoxycholate with leptospira. The protoplasm cylinder is damaged while the fibril retains its form.

DISCUSSION

The presence of a natural antileptospiral factor in milk found by us (Kirschner and Maguire, 1955) by dark-ground investigations and animal experiments has been confirmed by electron micrographs. The rapidity of the lytic action was remarkable. Micrographs taken 45 minutes after mixture of 1 ml. milk (diluted 1/2 with tap water) with a relatively large number of leptospira (2×10^6–2×10^7) showed the progress of the damage to their structure. It began with the damage to the protoplasm cylinder with the result that its second component, the fibril, which is wound around it, became clearly visible. After longer intervals up to 40 hours, the effects of destruction became more marked so that after this time only the fibril surrounded by masses of protoplasm could be seen.

This natural agent present in fresh milk did not change after pasteurization. There was no difference between the lytic action on virulent and non-virulent mutants of these organisms. This is in agreement with our animal experiments described in a previous paper (Kirschner and Maguire, 1955). Fresh milk mixed with virulent leptospira (containing several lethal doses) and kept for 3 hours at room temperature did not produce any symptoms in guinea-pigs, while the mixtures containing an equal number of organisms in tap water killed the animals with the typical changes of Weil's disease.

The lytic effect of immune antibodies in patient's and rabbit's immune sera (diluted 1/10–1/100) was quite different. There was much more damage to both components of the structure of leptospira. After 45 minutes compact clumps and small spheric granules were to be seen besides still completely normal organisms. After longer intervals no unlysed leptospira were found in these mixtures.

These findings are at variance with recent investigations by Lawrence (1955) who used a quite different technique. He first immobilized the organisms with agar before adding the immune serum and he came to the conclusion that leptospira in the "lysis balls" were not dead. This would contradict the term lysis, *i.e.*, destruction of organisms as repeatedly observed first by Schüffner and his associates and confirmed by many authors.

Owing to the different technique used by Lawrence our results cannot be compared. We used a modified method of Schüffner (using tubes in place of pallettes) and confirmed his observations that in certain dilutions complete lysis

of the organisms occurred. The lysis balls are the precursors of the spheric shining masses showing complete loss of the structure (lysis) of the organisms (Fig. 6).

The occurrence of cystic forms described by Swain (1955) has been observed by us especially in mixtures of immune sera with living leptospira. Only exceptionally were these forms seen in young (3–4 days old) cultures mixed with tap (rain) water used as controls (Fig. 7). It may be mentioned that they have also been found in some of the many micrographs taken at various intervals after mixing with milk.

The high rate of positive reactors (25–30 per cent) among cattle examined at random by us (Kirschner, observations in Dunedin, unpublished) and Te Punga and Bishop (1953), and the large percentage which dairy farmers constitute of the total of all cases of leptospirosis in New Zealand (80 per cent of 307 cases) indicate that there is a risk of infection associated with the work in this environment. Experimental infections of cattle (Reinhard, 1952 ; Webster and Reynolds, 1955) have shown that the animals after acute or sub-clinical infections were excreting leptospira in the urine for several weeks or months. This presented the possibility of human infections through the milk as in brucellosis. However, so far no milk-borne infections or epidemics have been recorded in many countries where bovine leptospirosis is known to be prevalent. This could be explained by the natural antileptospiral agent present in the milk.

SUMMARY

The presence of a natural antileptospiral agent in milk found by us in investigations under the dark-ground microscope and by animal experiments has been confirmed by studies with the electron microscope.

The electron micrographs showed that the lytic effect occurred rapidly: 45 minutes after mixing the milk with leptospira the protoplasm cylinder had been damaged while the other component of the organism, the axial filament, was unchanged. After 24 and 40 hours at room temperature only the axial filaments surrounded by protoplasm masses were seen.

There was no difference in the effect on virulent and non-virulent mutants or on different serotypes of leptospira.

The lytic effect of immune serum in certain dilutions forming lysis balls has been shown. Repeated attempts to cultivate leptospira from these shining spheric masses were not successful.

These investigations, forming part of our study of the epidemiology of human leptospirosis in New Zealand, may explain the absence of milk-borne infections in the many countries where bovine leptospirosis is prevalent.

Part of the work described in this paper was carried out at the Dominion Physical Laboratory of the Department of Scientific and Industrial Research, and is published by permission of the Secretary.

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THE DANYSZ EFFECT: AN INVESTIGATION OF THE REACTION BETWEEN AN ENZYME AND ITS ANTIBODY

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DANYSZ (1902) observed that a volume of antitoxin sufficient to neutralize a given dose of diphtheria toxin is insufficient to neutralize this dose if the toxin is added in two or more fractions with an interval of 15 minutes or more between each addition. This behaviour could not only be demonstrated *in vivo* but also *in vitro* (Schmidt, 1927, 1928) and with antigens other than diphtheria toxin. Heidelberger (1933) found it in the precipitin reaction of pneumococcus polysaccharide Type III, and Burnet (1931) in the interaction of staphylococcus toxin and antitoxin. Danysz deduced from his experiments that toxin and antitoxin could combine in more than one proportion and although his views were not accepted by his contemporaries the experiments of Heidelberger (1933) have confirmed them in general and those of Healey and Pinfield (1935) in particular.

The Danysz effect can be demonstrated in the reaction between the lecithinase of *Cl. welchii* (identical with α toxin, see Macfarlane and Knight (1941)) and horse antibody and can thus be examined *in vitro* in a three-component system of antigen, antibody and substrate (Cinader, 1952, 1955). For this reason and in the hope of obtaining a useful general method for the examination of enzyme-antibody interactions, the following study was undertaken. Another aspect such experiments may help to elucidate, that of the rôle of antibody as inhibitor, has recently been discussed elsewhere (Cinader, 1957).

In the following experimental analysis the Danysz effect was first examined in terms of the ability of antigen and antibody to combine in multiple proportions. This was found to account for experimental results in a qualitative, but not in a quantitative manner. The influence of a second factor, aggregation, was therefore explored in another series of experiments. When this second factor was considered in conjunction with the first a complete explanation of the Danysz effect and some conclusions about the nature of aggregation could be obtained.

MATERIALS AND METHODS

Purification of egg lecithin

Egg lecithin was prepared by a method similar to that of Macfarlane and Knight (1941). Yolks from twelve eggs were mixed with 150 ml. of 0·15 M-NaCl. The mixture (420 ml.) was extracted three times with 400 ml. of ethyl ether. The extracts (1220 ml.) were pooled, centrifuged and evaporated under reduced pressure to a volume of 300 ml. Acetone (700 ml.) was added to this solution and the precipitated material was removed by centrifugation and dissolved in 200 ml. of ethyl ether. The solution was freed from undissolved solids by

centrifugation, kept at — 20° for 20–30 min., and cleared by centrifugation and a further 800 ml. acetone were added. The precipitate was dissolved in 140 ml. ethyl ether, reprecipitated with 110 ml. acetone, separated by centrifugation, dissolved in 35 ml. ethyl ether, cleared by centrifugation, and reprecipitated again with excess acetone. The precipitate was then washed twice with acetone and dried *in vacuo* for 72 hr. The dried powder was weighed (yield 0·5 to 0·8 g. lecithin per egg yolk) and was made up with distilled water to a 16 per cent (w/v) emulsion of egg lecithin. A stable emulsion which did not separate into two phases was most readily obtained by adding water drop by drop to the lecithin powder, working it into a very smooth paste, freezing this paste several times in the course of further dilution and creaming the semi-solid sludge. Some experiments were carried out with a commercial lecithin preparation (Nutritional Biochemical Co., Cleveland).

Preparation and concentration of Cl. welchii *toxin*

Cl. welchii (Type A strain 107 ; N.C.T.C. No. 6125) was grown in meat infusion broth for 12 hr. and then removed from the broth by filtration through paper pulp. The filtrates were 2/3 saturated with $(NH_4)_2SO_4$. A solid phase separating as a scum was lifted off with a wooden spatula, pressed on unglazed plates of clay and was then dissolved in distilled water. The solution was centrifuged and the supernatant was dialysed against distilled water for 72 to 96 hr. and against 50 per cent glycerine for a further 72 hr. The toxin solution was stored at + 4°.

The toxin preparations used in the following experiments were characterized by the units of enzyme activity, units of combining capacity and nitrogen content of 1 ml. of each preparation (Table).

TABLE.—*Characterization of Enzyme Preparations*

Enzyme preparation	Units/ml.		mg. N/ml.
	Enzyme activity	Combining capacity	
4G	147	250	5·20
7A	345	667	0·95
6A	27	71	1·24

Immunization of horses

Bleedings were obtained from horses immunized with crude *Cl. welchii* toxin in the course of routine preparation of antitoxic sera. These bleedings are referred to by the name of the horse from which they were obtained (" Bassoon ", " Vermin ", " Beecham ").

Determination of nitrogen

Estimations were carried out by the micro-kjeldahl method. Selenium oxychloride was used as a catalyst.

Enzyme assays

Enzymic activity of *Cl. welchii* lecithinase was measured by the manometric method of Zamecnik, Brewster and Lipmann (1947). The potency of enzyme preparations was expressed (1) in terms of their catalytic action based on the volume of a preparation giving a constant activity, and (2) in terms of its combining capacity as an antigen based on the largest volume of a preparation of lecithinase completely inhibited (5–30 min.) by a standard preparation of antitoxin. Both determinations were carried out at 37° in a solution containing 5·1 per cent egg lecithin and 0·0039 M-$CaCl_2$.

The unit of enzyme activity was defined as that volume of a preparation which gave rise to the evolution of 50 µl. CO_2 between the 5th and 20th min. after the addition of enzyme to substrate.

The unit of combining capacity was contained in the largest volume of an enzyme preparation that was completely inhibited by an antiserum (Lister Institute Laboratory standard ESS No. 1) of which 0·0108 ml. was equivalent to an L+ dose of toxin. Varying volumes of

enzymes were incubated for 30 min. at 37° with 0·00108 ml. of antiserum ESS No. 1 in a total volume of 0·2 ml. The volume of CO_2 evolved between the 5th and 30th min. was measured.

Experiments on the Danysz effect: Two-stage addition of enzyme

Unless other details are given enzyme was added to antiserum in thin-walled test tubes (5 × 0·8 cm.) and the liquid was immediately mixed by 15 inversions of the stoppered tube. The tubes were kept in a thermostatic water bath and were immersed to about two-thirds the height of the column of liquid to bring about stirring through convection. After 30 min. a second quantity of enzyme was added, the solution was immediately mixed and aliquots pipetted into the side arm of Warburg flasks. The second period of incubation, usually 30 min., took place in the sac of a side arm at 37°, while the manometers were being filled with CO_2 and equilibrated. All mixtures contained the same amount of human albumin (1·4 per cent w/v) which was added to stabilize the enzyme and all the final reaction mixtures contained 0·09 M-$NaHCO_3$. Other experimental conditions were as follows:

Experiment 1 (*Fig.* 1).—A mixture was made consisting of enzyme preparation 4G and different quantities of antiserum "Bassoon". After incubation a further quantity of enzyme preparation 4G was added and 0·2 ml. of these mixtures were pipetted into side arms of Warburg flasks. Each side arm carried 0·04 ml. of preparation 4G. After addition of the side arm contents to the substrate, the reaction mixture had a volume of 2·2 ml. and contained 2·8 per cent of commercial lecithin and 0·004 M-$CaCl_2$.

Experiment 2 (*Fig.* 2).—Final concentrations as in Experiment 1. Enzyme added initially 25 and 75 per cent respectively of the final enzyme concentration.

Experiment 3 (*Fig.* 3).—Final concentrations of reactants other than antiserum as in Experiment 1 but the quantity of enzyme added initially was varied and the quantity of antiserum "Bassoon" kept constant for each experimental series at 0·0012, 0·0013, 0·0014, 0·0016 ml. respectively.

Experiment 4 (*Fig.* 4).—A Warburg flask was used with one side arm carrying a double sac. Enzyme preparation 6A mixed with immune serum was pipetted into one sac and enzyme preparation alone into the other. The contents of the two sacs were mixed with one another, the resulting solution having a volume of 0·4 ml. and containing 0·14 ml. of preparation 6A and 0·0032 ml. of serum "Vermin". One or 5 min. after this mixture was made, it was tipped into 1·8 ml. of a suspension of egg lecithin. The final reaction mixture contained 5·1 per cent (w/v) egg lecithin and 0·00391 M-$CaCl_2$.

Experiment 5 (*Fig.* 5).—After the second addition of enzyme, each side arm contained 0·028 ml. of enzyme preparation 7A and a constant volume of serum "Beecham" which was in one series 0·0028 ml., in a second series 0·00236 ml. and in a third 0·00264 ml. These reagents were contained in a volume of 0·2 ml. and were tipped into a volume of 2·0 ml. After this addition the reaction mixture contained 4·5 per cent (w/v) of egg lecithin and 0·0039 M-$CaCl_2$.

Experiment 6 (*Fig.* 6).—(*a*) Into the side arms of each Warburg flask were pipetted 0·2 ml. of supernatants prepared from mixtures of 0·028 ml. enzyme preparation 7A and varying quantities of serum "Beecham". (*b*) The side arm of each flask contained half the quantity of supernatants examined in (*a*) and 0·007 ml. enzyme preparation 7A. The final reaction mixtures contained 0·0039 M-$CaCl_2$ and 4·5 per cent (w/v) egg lecithin.

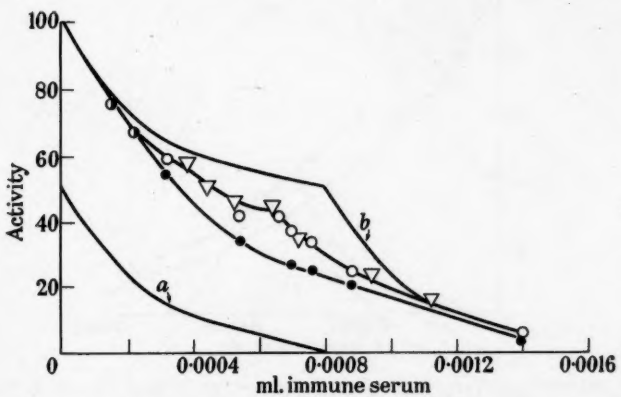
Experiment 7 (*Fig.* 7).—Mixtures of enzyme and antibody were kept in glass tubes; after incubation aliquots were incorporated in lecithin mixtures which were pipetted into Warburg flasks. The second quantity of enzyme was pipetted into the side arm of the flasks. Finally there were in each flask 0·0276 ml. of enzyme preparation 7A, 0·0236 ml. or 0·0296 ml. of serum "Beecham", 4·5 per cent (w/v) egg lecithin and 0·0039 M-$CaCl_2$.

Experiment 8. (*Fig.* 8).—Different quantities of enzyme preparation 7A were mixed with a constant quantity of "Beecham" antiserum. The resulting precipitate was removed by centrifugation and a second quantity of enzyme preparation 7A added to each supernatant; an aliquot of 0·2 ml. was pipetted into the side arm of a Warburg flask. Each flask contained 0·028 ml. of enzyme preparation 7A and 0·00236 ml. of serum "Beecham". After the contents of the side arm had been added the reaction mixture contained 4·5 per cent (w/v) egg lecithin and 0·0039 M-$CaCl_2$.

RESULTS

Observations in Antigen Excess

A neutralization curve of enzyme by antibody was obtained by adding increasing amounts of antibody to constant quantities of enzyme, incubating the mixtures for 30 min. at 37° and adding them to substrate. A plot of the enzymic activity of these mixtures as a function of the antiserum added was initially linear, but deviated from linearity as the volume of antibody was increased approaching the base line asymptotically (Macfarlane and Knight, 1941 ; Cinader, 1953*a*). If the same quantity of enzyme was added to antibody in two equal amounts with an incubation period of 30 min. after each addition, the neutralization curve showed a plateau. The activity of the mixtures obtained by the two-stage addition of enzyme was higher than that of the mixtures obtained by one-stage addition, except in extreme antigen excess (Fig. 1).



Scale of ordinate = activity as per cent of the activity of total enzyme in the absence of antibody.

FIG. 1.—The addition of a constant volume of enzyme to different quantities of immune serum.

All curves are based on mixtures containing 0·04 ml. of enzyme preparation 4G except the bottom curve (*a*), where the total enzyme is 0·02 ml. of preparation 4G.

—●— total enzyme added at once followed by an incubation of 30 min.

—○— half the total quantity of enzyme was added initially. After 30 min. incubation (37°) the second half of the enzyme was added. The mixture was incubated for a further 30 min. before addition to substrate.

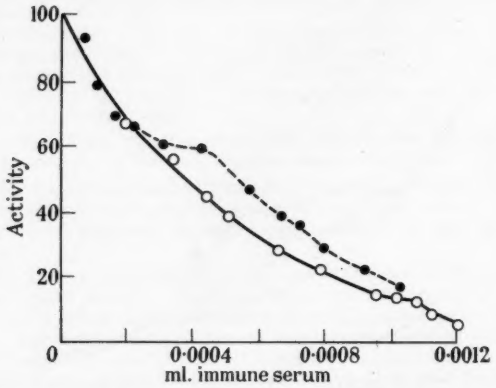
—▽— half the total quantity of enzyme was added initially. After 60 min. incubation (37°), the second half of the enzyme was added. The mixture was incubated for a further 30 min. before addition to substrate.

b construction line (for explanation see text).

The conditions prevailing after the first addition of enzyme were observed by measuring the activity of mixtures containing 50 per cent of the enzyme and variable amounts of antibody. From this neutralization curve a neutralization curve of the two-stage addition may be constructed by assuming (1) no antibody is available for combination with a second quantity of enzyme in any mixture containing less than the neutralizing dose of antibody. (2) antibody in excess

of the neutralizing dose remains uncombined and is therefore available for combination with enzyme added at the second stage.

A curve constructed on these assumptions agrees qualitatively with the experimental neutralization curve of two-stage addition up to the plateau in the curve but had a much steeper slope than the experimental curve for concentrations of antibody approaching that required for complete inhibition. Less antibody is combined with the first quantity of enzyme added than would be expected from the second assumption. It would seem that the first of the two assumptions may be correct. As a consequence the position of the plateau of neutralization curves obtained by addition in two stages of enzyme to antibody should depend on the relative quantity of the two additions. This was found to be the case (Fig. 2).



Scale of ordinate = activity as per cent of the activity of total enzyme in the absence of antibody.

FIG. 2.—The addition in two different amounts of a constant volume of enzyme to different quantities of immune serum.

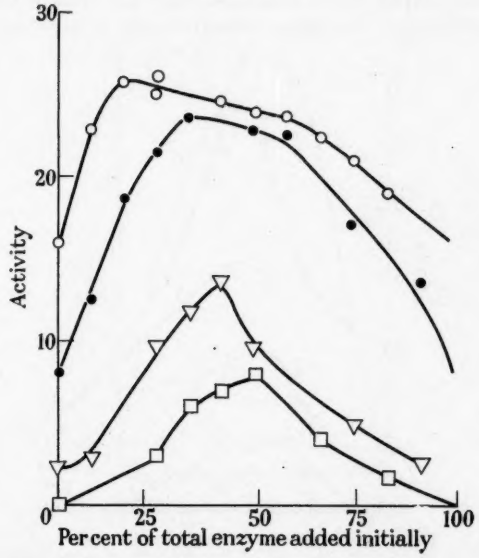
Total enzyme added in 0·2 ml. : 0·04 ml. of preparation 4G.
After each addition the mixtures were incubated for 30 min.
—●— first addition 25 per cent of total enzyme, second 75 per cent.
—○— first addition 75 per cent of total enzyme, second 25 per cent.

The results shown so far have established that the Danysz phenomenon can be demonstrated over a considerable range of antigen excess, that it depends on the shape of the neutralization curve in the initial but not in the final part of this range and that the residual activity depends on the proportion of total enzyme added first.

Observations in Antibody Excess

Varying quantities of enzyme were added to constant quantities of antibody, the mixtures incubated for 30 min. at 37°, and a second amount of enzyme added to make the final concentration of enzyme in all the mixtures the same. After a second period of incubation (30 min., 37°) aliquots of the mixtures were added to substrate and their activity was measured. The activity was plotted

as a function of the percentage of total enzyme added first (Fig. 3). The curves obtained in this manner were bell-shaped, their maxima of enzyme activity depending on the quantity of antibody. A quantity of antibody which gave minimum activity in neutralization experiments (Fig. 1) led to a curve with its maximum corresponding to an initial addition of 50 per cent of the final enzyme concentration ; smaller quantities of antibody led to curves of which the maxima were displaced to lower values of enzyme initially added. With larger quantities of antibody it was not possible to determine the position of the maxima since the measured activities were within the experimental error.



Scale of ordinate = μl. CO_2 (10th–75th min.) after addition and incubation of second quantity of enzyme.

FIG. 3.—Two-stage addition of a constant volume of enzyme to constant volumes of antibody.

The concentration of reactants is adjusted so as to avoid precipitation during the course of the experiment. Every mixture contained 0·04 ml. of enzyme preparation 4G in the aliquot measured. Each of the curves corresponds to a different constant volume of antiserum, showing the effect of the percentage of enzyme added initially on the enzymic activity of the final mixture.

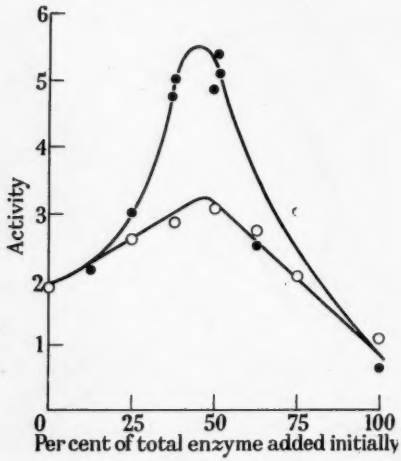
Period of incubation after each addition : 30 min.

—○— 0·0012 ml. serum " Bassoon ".
—●— 0·0013 ml. „ „
—▽— 0·0014 ml. „ „
—□— 0·0016 ml. „ „

The activity of the mixtures decreased when the second period of incubation was increased to 1 hr. ; shorter periods of incubation would thus be expected to show maxima closer to expected values. Attempts were therefore made to measure activities after a short second incubation.

After the first addition of enzyme to the antibody, aliquots of the mixtures were pipetted into one sac of Warburg flasks which had one side arm equipped with a double sac. The second quantity of the enzyme was pipetted into the second sac. The flasks were shaken in the water bath for 30 min. at 37°, the solutions in the two bulbs were then mixed, and the mixture was tipped into the substrate solution 1 min. and 5 min. after mixing. The maxima of these curves were approximately 6 and 3 per cent of the activity of the total enzyme in the absence of antibody (Fig. 4).

When the reaction described was carried out with higher absolute concentrations of antigen (see Experiment 5) and a neutralizing dose of antibody, the maximum of the bell-shaped curve was less than 5 per cent of the enzyme activity in the absence of antibody. At high concentrations of antibody there were some



Scale of ordinate = activity as per cent of the activity of total enzyme in the absence of antibody.

FIG. 4.—Two-stage addition of enzyme to a constant quantity of antiserum.

Effect of the duration of the second period of incubation.

Each mixture contained 0·14 ml. of enzyme preparation 6A and 0·0032 ml. of antiserum "Vermin".

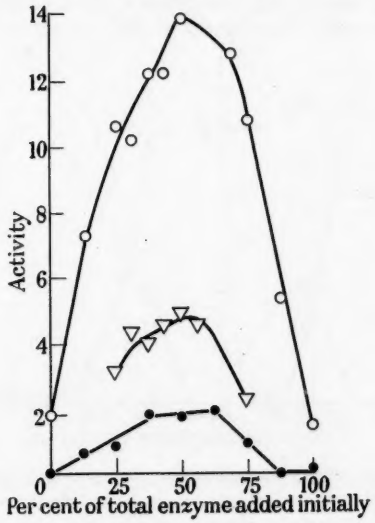
—●—First incubation 28 min., second incubation 1 min.

—○— „ „ „ „ „ 5 „

slight indications that the maximum of the bell-shaped curve was shifted to values corresponding to amounts greater than 50 per cent of the total enzyme added initially (Fig. 5). This observation was fully confirmed in experiments to be described later (Fig. 7).

The results so far described could be accounted for qualitatively, if not quantitatively, by the ability of antigen to bind twice as much antibody as is required for neutralization. Were a single antigen being considered, this fact could be tested by nitrogen assays of immune precipitates; with an impure antigen this is impossible. It was therefore necessary to obtain information

by the continued use of a method which employed a unique property of the antigen, the catalytic activity. To this end constant quantities of enzyme were mixed with different quantities of antibody and kept for 1 hr. at 37°. The resulting precipitates of antigen and antibody were removed by centrifuging and the enzyme activity of the supernatant was assayed. A constant quantity of enzyme was subsequently added to an aliquot of each of these supernatants and the activity of the resulting mixture was measured. These activities are plotted in Fig. 6 as a function of the volume of serum contained in the mixtures from which the supernatants were derived. The supernatants show a decrease of activity with increasing volume of serum and a well-defined composition at which



Scale of ordinate = activity as per cent of the activity of total enzyme in the absence of antibody.

FIG. 5.—Two-stage addition of enzyme to constant quantities of antibody.

Three groups of mixtures were chosen: one in slight antigen excess (0·00236 ml. serum "Beecham"), another near neutrality (0·00264 ml. serum) and a third in antibody excess (0·0028 ml. serum). Every mixture contained in the aliquot measured 0·028 ml. of enzyme preparation 7A.

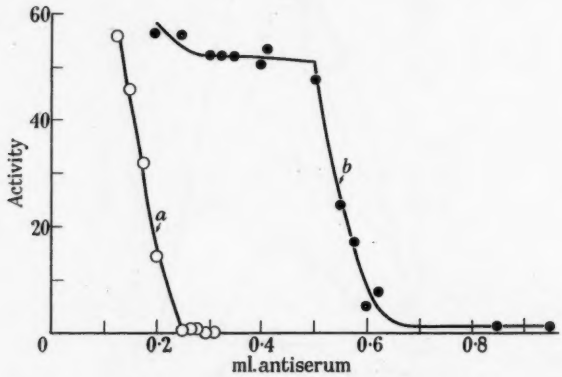
First incubation 30 min., second incubation 30 min.

—○— 0·00236 ml. serum.
—▽— 0·00264 ml. ,,
—●— 0·0028 ml. ,,

no further enzyme activity remains after removal of floccules. A similar plot for the supernatants to which enzyme had been added shows a plateau and a sudden decrease of activity at a concentration of antibody twice the concentration in the mixtures in which no residual enzyme activity can be demonstrated after removal of the precipitate. It follows that the proposition under examination is likely to be correct and that antigen can bind twice the amount of antibody

necessary for neutralization. If this were to be the explanation of the shape and height of the plots of enzyme activity against per cent of enzyme added initially to a constant amount of antibody, the height of such curves at the maximum would be expected to be half the activity of the total enzyme. This, however, is clearly not the case. A different experimental attack was therefore required and the following investigation was designed to explore the cause of this crucial discrepancy.

It seemed likely that the activities observed in the foregoing experiments, even after a second incubation of only 1 min. (Fig. 4) represented a nearly complete " equilibration " of the complex of enzyme and antibody formed during the first incubation with the free enzyme added subsequently. Such a " reversal " could be delayed by making use of the observation that enzyme and antibody reach a different equilibrium when combining in the presence of substrate and when



Scale of ordinate = activity as per cent of the activity of a standard quantity of enzyme.
Scale of abscissa = ml. antiserum in each ml. of the mixture from which the supernatants (*a*) were prepared.

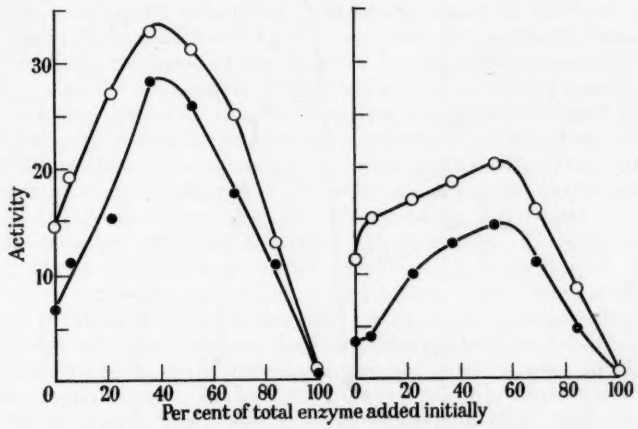
FIG. 6.—Demonstration of the relative quantities of antibody bound in a neutral complex and in excess antibody.

a. The activity of supernatants from mixtures containing the same quantity of enzyme and varying amounts of antibody after removal of the precipitated complex of antigen and antibody. Every mixture contained in the aliquot measured 0·028 ml. of enzyme preparation 7A (that is 0·14 ml. per ml. of the original mixture of antigen and antibody).

b. The activity of supernatants from *a*. after the addition of 0·007 ml. of enzyme preparation 7A.

combining in its absence (Zamecnik and Lipmann, 1947 ; Cinader, 1953*a*) and that this equilibrium is reached very rapidly. Experiments based on these facts could also indicate the relative position of the maximum of the plot of residual enzyme activity as a function of the percentage of antigen added initially when the dose of serum is in excess of the neutralizing dose and when it is equal to it. Since the mixtures of antigen and antibody in the first stage of the experiment contain considerable excess of antibody and are therefore enzymically inactive, they can be allowed to remain in contact with the lecithin mixtures during the period of gassing and equilibration.

Mixtures of different amounts of enzyme and constant amounts of antibody were kept at room temperature overnight. Subsequently aliquots of these mixtures were incorporated in the lecithin suspension and pipetted into Warburg flasks. Enzyme was added to the side arms in such quantities as to bring the total enzyme in each flask to the same concentration. After 30 min. at 37° the contents of the side arms were tipped into the flask and the amount of CO_2 evolved was measured. The data, plotted in the usual way (Fig. 7), showed that the maximum was shifted to higher values of enzyme initially added as the amount of antibody added was increased from the neutralizing to a larger dose. Furthermore the height of the maximum of the curve corresponding to the neutralizing dose of antibody was initially 33 per cent and decreased to 28 per cent in the following period of observation. An element of asymmetry was



Scale of ordinate = activity as per cent of total enzyme in the absence of antibody.

FIG. 7.—Two-stage addition of enzyme.

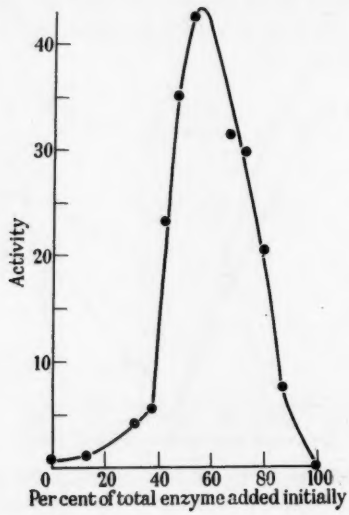
The mixture of the first quantity of enzyme and antibody was added to substrate; the second quantity of enzyme was added to the resulting mixture. The final quantity of enzyme preparation 7A in each mixture was 0·0276 ml. The activity of the mixture was measured at two intervals.

—○— 0–5 min.
—●— 10–20 min.

The serum dose was 0·0236 ml. in the experiment shown on the left side of the graph and 0·0296 ml. in the experiment shown on the right side.

introduced into the curves by the fact that " reversibility " was most pronounced in mixtures which contained the smallest quantities of enzyme during the first incubation. Nevertheless " reversibility " was observed even when the initial dose of enzyme was in excess of half the final concentration and could be expected to have bound all the antibody. This would suggest that enzyme added to a complex of antigen and antibody can combine directly with this complex. Before this conclusion could be accepted, it had to be demonstrated that the removal of the immune precipitate after the first addition of enzyme and the addition of the second quantity of enzyme to the supernatants would result in mixtures

of which the most active would show 50 per cent of the activity of enzyme in the absence of antibody, even after prolonged incubation. Varying quantities of enzyme and antibody were incubated at room temperature overnight and then centrifuged. Enzyme was added to aliquots of the supernatants so as to adjust the concentration of enzyme in all mixtures to the same value. After incubation for 30 min. at 37° aliquots of these mixtures were tipped from side arms into the lecithin mixture of Warburg flasks. The maximum of the resulting plot, based on a measurement between the 10th and the 20th minute after addition, was 48 per cent of that of enzyme activity in the absence of antibody and thus confirmed the suggestion that " reversibility " must be attributed to combination of enzyme with the complex between enzyme and antibody (Fig. 8).



Scale of ordinate = activity as per cent of total enzyme in the absence of antibody.

FIG. 8.—The activity of mixtures of enzyme, added in two stages to antibody—the effect of removal of the precipitate of antigen-antibody before the second addition.

Activity measured between the 10th and 20th minute after addition of substrate. Each mixture contained 0·028 ml. of enzyme preparation 7A and 0·00240 ml. serum " Beecham ".

DISCUSSION

By the addition of two equal quantities of enzyme to varying amounts of antiserum it was shown that the Danysz effect may be demonstrated with *Cl. welchii* lecithinase and equine lecithinase antibody far into the zone of excess antigen. A neutralization curve representing the activity of mixtures to which enzyme had been added in two equal stages could be accounted for in the region of low antibody concentration by the inability of antibody already combined with enzyme initially added to combine with further enzyme (Fig. 1). For higher antibody concentrations the course of the curve could not be adequately explained and a different experimental arrangement was chosen to investigate

this problem and to gain insight into the Danysz effect in mixtures containing excess antibody.

The Danysz effect in the reaction between diphtheria toxin and antitoxin was examined by Healey and Pinfield (1935) in terms of the fractional addition of toxin to a constant amount of antitoxin. Their results were similar to those obtained in the reaction of enzyme and anti-enzyme and their discussion of the mechanism of the Danysz phenomenon can serve as a starting point of a consideration of experiments presented in this paper.

Mixtures of enzyme and antibody, containing identical concentrations of reactants and differing only in the percentage of total enzyme added in the first of two additions, differed in their enzymic activity. The activity of the mixtures was plotted as a function of the fraction of enzyme added initially (Fig. 3, 5). The shape of the resulting curves and the shift of their maxima corresponding to different quantities of antibody could be derived by assuming that a compound of composition EA_n (E = enzyme, A = antibody) was completely inactive, that EA_{2n} represented the limit of the combining capacity of enzyme (or that EA_{2n} was the only stable compound of high antibody content) and that the compound predominantly formed was the one which allowed the majority of both antigen and antibody molecules to combine. If the final concentration of enzyme and antibody corresponded to the compound EA_n, the initial addition of 50 per cent of the enzyme would give rise exclusively to the formation of the compound EA_{2n}, would thus bind all the antibody and would not leave any antibody free to react with enzyme added subsequently. If, however, less than 50 per cent of the enzyme were added initially, the formation of EA_{2n} would result in some antibody remaining uncombined. This free antibody could subsequently combine with more enzyme. Whilst the first addition of enzyme led to a mixture containing excess antibody, the subsequent addition of enzyme led to a mixture which contained excess antigen with respect to the antibody available for combination. As a consequence, enzyme introduced during the second addition would form the inactive compound EA_n and the available antibody would combine with twice as many molecules of enzyme as were bound by a molecule of antibody in the first stage of the experiment. The final activity of the mixture to which less than 50 per cent of the enzyme was added initially would be less than that to which enzyme was added in two equal amounts ; the smaller the percentage of enzyme added initially, the smaller the resultant activity of the mixture after the final concentration of enzyme was made up to 100 per cent.

If more than 50 per cent of the total enzyme were added initially all the antibody would combine, giving rise to an enzymically inactive mixture of EA_n and EA_{2n}, and none of the antibody would remain free to combine with enzyme added subsequently. In these conditions the activity of the mixtures would be equal to that of the second quantity of enzyme added and would therefore be smaller the greater the initial addition. The assumptions made thus lead to the conclusion that the highest enzyme activity would be expected to be half that of the total enzyme and would be observed in a mixture made by the initial addition of half the final enzyme concentration.

So far we have considered mixtures containing a quantity of antibody which was the smallest amount neutralizing the total enzyme. The conclusions reached can be tested by examining fractional addition of the same total amount of

enzyme to a different quantity of antibody. It can be deduced that the position and height of the maximum in relation to the percentage of total enzyme added initially, would be governed by the quantity of antibody. It would correspond to an initial addition of antibody which would result in the combination of all the antibody and of all the antigen as the compound EA_{2n}. If the quantity of antibody were x per cent of the neutralizing dose, the maximum would be $100 - \frac{x}{2}$ per cent of the activity of the total enzyme and would be found in a mixture to which $\frac{x}{2}$ per cent of enzyme had been added initially.

One of the basic assumptions of the foregoing analysis implied that the stable compound of highest antibody content contained twice as much antibody per molecule of enzyme as the compound formed at neutrality. The validity of this assumption was confirmed when it was found by addition of enzyme to supernatants that virtually all antibody was removed with the precipitates of antigen and antibody from such mixtures as did not exceed twice the content in antibody of the enzymically inactive mixture of lowest enzyme concentration (Fig. 6). Though this observation did not exclude the existence of soluble compounds containing more antibody than does the compound EA_{2n}, it justified the examination of other experimental evidence in the light of the assumption that EA_{2n} is the stable compound of highest antibody content.

Measurements of the residual activities of mixtures to which enzyme was added in two quantities were plotted as a function of the percentage of enzyme added initially and led to curves which agreed with expectation in several details; for a neutralizing dose of antiserum the maximum corresponded to a mixture to which enzyme was added in two equal amounts. When the amounts of antiserum were less than the neutralizing dose the maximum was progressively shifted to mixtures to which less than 50 per cent of enzyme were added initially (Fig. 3, 7). The relative shift of the maximum for amounts of antiserum in excess of the neutralizing dose could be demonstrated only if the second quantity of enzyme was added to the first mixture of enzyme and antibody in the presence of substrate (Fig. 7). In these conditions each curve is distorted and its maximum is displaced since the presence of substrate during the second addition affects the activity of mixtures in antigen excess to a less marked degree than mixtures in excess antibody (Cinader, 1953*a*); nevertheless two curves corresponding to two different doses of antiserum may be compared with respect to the relative position of their maxima. By comparison with mixtures containing a neutralizing dose of antiserum it was found that mixtures of highest enzyme activity corresponded to a larger initial addition of enzyme when the antibody content of the mixtures exceeded the neutralizing dose.

The position of the maxima was examined with a number of enzyme preparations which differed in the ratio between combining units and activity units and was found to be independent of this factor. The rôle of inactive enzyme molecules may therefore be omitted from this discussion (see also Cinader, 1953*b*).

While the experimental data discussed so far agreed qualitatively with the conclusions which are being tested, they did not agree in one important quantitative aspect. The activity of mixtures to which enzyme had been added in fractional amounts was far lower than expected. The activity of enzyme added in two equal amounts to a neutralizing dose of antiserum would be expected to

be half that of the enzyme in the absence of antibody; it was in fact 3–4 per cent (Fig. 5). A decrease of the second period of incubation to 1 min. did not appreciably affect this discrepancy (Fig. 4); only if the combination of the second quantity of enzyme was inhibited by the simultaneous addition of substrate did the mixtures display activities approaching the expected value; but even in these circumstances the initial activity was only 33 per cent of that of the enzyme in the absence of antibody (Fig. 7).

The assumptions so far made have implied that the complex between antigen and antibody formed in the course of the first addition of enzyme was no longer able to combine with antigen. This view might be justifiable in terms of the lattice hypothesis (Marrack, 1938; Pauling, 1940). Large symmetrical particles constructed as three-dimensional lattices of antigen and antibody would leave only antibody at the periphery of each particle free to combine with enzyme. This view has to be revised since the reaction obeys quantitatively the deductions under examination if the complex of antigen and antibody is removed before the second addition of enzyme (Fig. 8). It is therefore possible to proceed with the conclusion that the "reversibility" of the Danysz effect is brought about by the interaction between antigen and the complex of antigen and antibody.

Even under conditions of simultaneous addition, 33 per cent of the combined antibody reacted with enzyme and this would appear to be the lowest estimate of fraction of combined antibody available for immediate combination. Only small or very asymmetrical polymer particles would allow most of the combined antibody to react instantaneously with enzyme (see Landsteiner, 1945; p. 254). An aggregation less specific than that proposed by the lattice hypothesis would clearly provide much less impediment for combination of enzyme with a compound EA_{2n} (for instance: Bordet, 1920; Boyd, 1942). A decision between these two possibilities is outside the experimental scope of this paper. Light-scattering data have indicated an increase in particle size on addition of antigen to mixtures of antigen and antibody and may help to decide between these alternatives (Gitlin and Edelhoch, 1951).

Our concern in the present context is with the rôle of aggregation in the Danysz phenomenon. While combination of antigen and antibody in multiple proportions constitutes its underlying reaction, it would clearly be impossible to observe it without a restrictive mechanism which slows down the combination of a compound such as EA_{2n} with E resulting in a compound E_2A_{2n}, and we can assume that the restrictive mechanism is aggregation. It is now also possible to qualify the statement that EA_{2n} is the "stable" compound of highest antibody content. Stability can be taken to imply a high degree of aggregation. Compounds of antibody content in excess of EA_{2n} could exist without playing a part in the Danysz effect if they were not aggregated or were aggregated in such a way as would enable them to react immediately with antigen. It may finally be suggested that differences in the extent of the Danysz effect displayed by different sera may be attributed to strength or reversibility of aggregation.

SUMMARY

When lecithinase of *Cl. welchii* was added in two quantities to equine lecithinase antiserum the Danysz effect was observed in excess antigen and in excess antibody.

Enzyme activity was measured in mixtures which contained identical final concentrations of enzyme and different concentrations of antibody. When enzyme was added in two fractions there was a steep initial decrease, a levelling out, and a second steep decrease.

When the mixtures contained the same final concentration of enzyme and of antibody, a plot of enzyme activity as a function of enzyme added initially was bell-shaped. The most active mixture was found to have 3–4 per cent of the activity of the total uninhibited enzyme. If substrate was added after the first addition of enzyme and before the second, the activity was 33 per cent, and if the precipitate of antigen and antibody was removed before the second addition of enzyme the activity of the most active mixture was approximately 50 per cent of the activity of total enzyme.

The observations are accounted for in terms of the composition of compounds formed in neutral mixtures and in mixtures containing excess antibody and in terms of aggregation of such compounds. The Danysz effect is shown to depend not only on combination of antigen and antibody in multiple proportions but also on a second reaction which restricted combination of compounds containing antibody with further antigen.

This second reaction can be accounted for as the aggregation of the initial compound of antigen and antibody. The results would be in agreement with the existence of an entirely non-specific aggregate or of a non-specific aggregate of small or asymmetrical polymer units.

Thanks are due to Mr. B. Weitz, M.R.C.V.S., for sera from horses immunized with *Cl. welchii* lecithinase, to Dr. M. E. Mackay for a preparation of human albumin and to Mrs. V. Austin and Mr. B. Jordan for technical assistance.

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IMMUNE-ADHERENCE OF BACTERIOPHAGE T2

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BACTERIA sensitized with specific antibody and all four components of complement will attach themselves to human red blood cells at 37°. This phenomenon was described by Nelson in 1953 and called the immune-adherence phenomenon (I-A). Nelson (1953, 1956) demonstrated the occurrence of immune-adherence with many different species of bacteria, with rickettsiae, with particulate polysaccharide antigens (*e.g.*, starch granules) and with one virus, vaccinia. The degree of immune-adherence was measured either by counting adherent antigen particles or organisms directly under the microscope, or by determining the percentage of organisms removed from a suspension after incubation with human erythrocytes for a period at 37°, which were then deposited by light centrifugation. Subsequently, he found that immune-adherence could be measured by the formation of agglutination patterns by the clumped red cells settling in the bottom of a tube. It was thus possible to study immune-adherence of the smaller viruses, such as poliovirus, which Nelson and Woodworth (to be published) have shown to produce haemagglutination patterns with human erythrocytes in conditions suggesting strongly an immune-adherence mechanism. Confirmation of immune-adherence by small viruses is difficult because the number of particles adherent to red cells cannot be counted visually, and neither adherent virus nor residual virus in the supernatant fluid can be assayed by ordinary methods because the virus has been exposed to antibody. This difficulty could be overcome by labelling the virus with a radioactive isotope, and for this purpose the most suitable virus seemed to be bacteriophage. This paper describes experiments with bacteriophage T2 labelled with a radioactive isotope, which demonstrated that the fully sensitized virus adhered to the red cell surface, a result conforming exactly with Nelson's immune-adherence phenomenon. From this one concludes that haemagglutination obtained with phage T2 and other small viruses sensitized with antibody and complement is probably also an expression of immune-adherence.

MATERIALS AND METHODS

Virus.—T2 (wild type) stocks were prepared in bacteria growing in M9 synthetic medium (Delbrück, 1940) with *Bacterium coli* strain B as host. Phage was purified by three cycles of differential centrifugation at 4000 r.p.m. on an MSE angle centrifuge for 15 min. and at 14–20,000 ***g*** for 1 hr. on the Spinco centrifuge. It was re-suspended in phosphate buffer and had a titre of 1–5 $\times$ 10^{11} plaque-forming particles per ml. titrated on *Bact. coli* B.

^{35}S-labelled phage was prepared in bacteria growing in M9 synthetic medium with $MgCl_2$ substituted for $MgSO_4$ and 50 μc per ml. of carrier-free ^{35}S added. It was also purified by three cycles of differential centrifugation. The stock was absorbed with a variant of *Bact. coli* B resistant to phage T2 (B/2), and with washed red cells.

Radioactivity was measured by counting disintegrations per minute on an Ekco scaler, type N529, using dried samples sufficiently small to avoid loss by self-absorption.

Antiserum.—Antiserum was prepared by Dr. E. W. McCloy in a rabbit by repeated intravenous injections of broth culture T2 lysate over a period of six weeks. The antiserum had a K value of 2000 per min., measured at a serum dilution of 2×10^{-4} (Adams, 1950). Before use in I-A experiments, serum, usually diluted 1/4, was absorbed twice for 30 min. at 37° with 10^9 *Bact. coli* per ml. of serum. Serum was inactivated at 56° for 30 min.

Diluent.—Veronal buffered saline (Fulton and Dumbell, 1949) containing 0·5 per cent crystalline bovine plasma albumin was used in all I-A experiments.

Complement.—Guinea-pigs were bled by cardiac puncture and the fresh serum was pooled and stored at − 70°. Before use, complement diluted 1/4 was absorbed with 10^9 *Bact. coli* B per ml. and when necessary the complement was also absorbed with packed human red cells. Complement absorptions were done at 0°.

Red blood cells.—Group O Rh+ human blood in acid-citrate-dextrose solution was obtained from the National Blood Transfusion Service. Blood was stored at 4° and discarded a month after it was drawn. Red cells were washed three times in veronal buffer before use.

Glassware.—All glassware was acid cleaned. Kahn tubes were sterilized covered with aluminium caps.

I-A tests by haemagglutination patterns.—Antigen suspension in volumes of 0·5 or 1 ml. were mixed with 0·2 ml. volumes of antiserum dilutions in Kahn tubes. Volumes of 0·1 ml. of complement, usually diluted 1/16, were added and the tubes shaken and placed in a 37° water-bath. After 10 min., 0·1 ml. of a 1·5 per cent suspension of red cells was added to each tube. The tubes were shaken and incubated for 45–60 min. It was important to take readings at this time when the cells would have settled to the bottom and agglutinated cells would not have begun to slip. I-A haemagglutination is not as strong as that caused by influenza virus, for example; the pattern is easily disrupted and little or no clumping of the cells is visible under the microscope.

Controls.—The following controls, which must be negative, were included in every test: antigen, antiserum and complement each alone with red cells, and red cells alone in diluent. As a control of complement activity, a tube was also included containing a standard suspension of a particulate antigen, usually *Amaranthus cruentus* starch, sensitized with antibody and known to undergo I-A. A dilution of complement was chosen which would give maximum I-A but which did not itself agglutinate red cells. It was often necessary to absorb human red cell agglutinins from guinea-pig serum to obtain such a dilution.

I-A tests with labelled T2.—These experiments were made by incubating reaction mixtures which included dilutions of labelled T2 suspension, antibody, complement and red cells, the quantities depending on the experiment, for 1 hr. at 37° and then centrifuging the mixture to deposit the red cells. The supernate was withdrawn; the red cell pellet was washed once and re-suspended in 0·5 ml. of diluent. Samples from the supernate and pellet were dried and radioactivity assayed. The numbers of disintegrations in the pellet were expressed as percentages of total disintegrations in the reaction mixture.

RESULTS

Haemagglutinating properties of complement-phage mixtures

Neither T2 nor antiserum nor a mixture of them agglutinated human red cells. Complement, which was always first absorbed with human red cells, did not by itself agglutinate human red cells either, but when 0·1 ml. of it was mixed with 0·5 ml. of T2 at 10^8 particles/ml., haemagglutination was produced in the absence of antiphage serum. As the complement had already been absorbed with human red cells, this effect was not due to antibody in the guinea-pig serum against human red cells. It was more probably due in part to normal antibody against *Bact. coli* because a further absorption with *Bact. coli* rendered the complement usable at dilutions greater than 1/4. It is known that bacterial antigens in solution can undergo immune-adherence (Turk, to be published); it is very probable that dissolved *Bact. coli* antigens in the phage lysate would

react with any " normal " antibody there might be in the complement, to give immune-adherence, and hence haemagglutination, not due to the phage.

Lysates containing 10^{11} and 10^{10} particles per ml. gave haemagglutination in absence of antiserum with some samples of complement, even after two absorptions with *Bact. coli*. Some antibody to *Bact. coli* may have remained in the complement even then, since it is notoriously difficult to remove normal antibody by absorption ; but some of the haemagglutinating activity of complement and T2 mixtures may have been due to normal antibody to the phage rather than to the bacterium.

Evidence that low dilutions of complement may induce immune-adherence of T2 by virtue of normal anti-phage antibody in it came from the following experiments : Serial twofold dilutions of fresh guinea-pig serum in 0·1 ml. amounts were mixed with 0·4 ml. of labelled T2 at 1×10^9 particles/ml., 0·5 ml. of diluent and 0·5 ml. of a 10 per cent suspension of red cells. The mixtures were incubated for 1 hr. at 37° and the radioactivity in the supernate and pellet assayed in the usual way. Table I gives the result of this titration.

TABLE I.—*Immune-adherence of Labelled T2 in the Presence of Low Dilutions of Complement and the Absence of Antiserum*

Reaction mixture:
- T2 : 4×10^8 plaque-forming particles in 0·1 ml.
- Complement dilutions : 0·1 ml.
- Diluent : 0·5 ml.
- R.B.C. $4{\cdot}5 \times 10^8$ in 0·5 ml.

Complement : final dilution	Percentage of total activity in pellet
—	1
1/15	70
1/30	58
1/60	28
1/120	11
1/240	2
1/480	<1

Thus normal guinea-pig serum induced adherence of T2, and a 50 per cent end-point was obtained between 1/2 and 1/4 initial serum dilution and between 1/30 and 1/60 final dilution. Fresh human serum was found to have an end-point at a similar dilution. In addition, both normal guinea-pig and human sera inactivate phage T2.

In spite of the anomalies introduced by presumed normal antibodies in the complement, it always proved possible by suitable absorption with human red cells and with *Bact. coli* to produce a complement giving satisfactory negative controls when used at initial dilutions greater than 1/4 and having a satisfactory immune-adherence titre (32–64) against known antigen/antibody systems.

I-A haemagglutination with T2

Table II illustrates a representative I-A chessboard titration of T2 against its antiserum. Controls of all reagents alone and in pairs were negative. Haemagglutination only occurred in tubes which contained T2, antibody and complement, and at the dilutions shown in the Table. No haemagglutination occurred with rabbit or sheep red cells.

TABLE II.—*Chessboard Titration of Phage T2 Against T2 Antiserum by I-A Haemagglutination Patterns*

Antiserum : final dilution	Plaque-forming particles of T2 per ml. 10^9	10^8	10^7	10^6	0
1/90	4	3	2	0	0
1/180	4	3	1	0	0
1/360	4	3	1	0	0
1/720	4	3	0	0	0
1/1440	4	2	0	0	0
1/2880	4	0	0	0	0
1/5760	0	0	0	0	0
0	0	0	0	0	0

Each tube contained 0·5 ml. of T2 dilution or diluent, 0·2 ml. of antiserum dilution, 0·1 ml. of complement diluted 1/16, and 0·1 ml. of 1·5 per cent suspension of R.B.C.
4 = very strong haemagglutination.
3 = strong haemagglutination.
2 = definite haemagglutination.
1 = weak haemagglutination.
0 = no haemagglutination.

Similar results of titrations were obtained on many different occasions using two different antisera, many samples of complement and different batches of red cells. The kind of haemagglutination and general results of these titrations were the same as those observed by Nelson with other antigens. Haemagglutination was obtained in experiments in which a total of $1{\cdot}5 \times 10^7$ red cells were used when more than 5×10^6 total phage particles were present. It therefore appears that haemagglutination could only be obtained when the ratio of plaque-forming particles to red cells was of the order 1 : 1. The ratio of plaque-forming particles to antigenic particles was not known ; it seems probable that haemagglutination requires more than one sensitized antigen particle adherent to each red cell.

I-A with phage labelled with ^{35}S

In order to show that labelled T2 particles immune-adhered and were carried down with the red cells, a difficulty had to be overcome. This was, that phage at a final concentration of 10^9 particles per ml., when incubated with antiserum at the concentration necessary for I-A, but without complement, would not remain suspended during centrifugation, whether red cells were present or not. In such experiments, there was no haemagglutination, but 40–60 per cent of the radioactive phage was found in the very slight deposit after centrifugation, even after very light centrifugation at 540 r.p.m. for 10 min. on an MSE angle centrifuge. The most probable explanation is that phage at this high concentration was being agglutinated by the antibody, although no flocculation could be seen. Concentrations of phage less than about 10^8 particles per ml. could not be used, because the proportion of labelled particles was inevitably small, and more dilute phage gave insufficient " counts " to permit a valid reading. For instance, a phage stock containing $6{\cdot}5 \times 10^{10}$ plaque-forming particles per ml. gave only $1{\cdot}7 \times 10^5$ counts per min. per ml., or $2{\cdot}6 \times 10^{-6}$ counts per plaque-forming particle.

However, it was possible to find a concentration of phage low enough to avoid deposition due to agglutination yet still high enough for assay by radioactivity. Lanni and Lanni (1953) stated that collisions between phage particles can only

be ignored at concentrations of 10^7 particles per ml. or less. For the purpose of experiments like that shown in Table III, collisions did not matter so long as, in the time occupied by the experiment, they were insufficient to cause deposition on centrifugation : controls showed that this condition was satisfied.

TABLE III.—*Demonstration of Adherence of Labelled Phage Particles to Red Cells in the Presence of Antibody and Complement*

Plaque-forming particles of T2 per ml.	Antiserum*	Complement*	R.B.C. per ml.	Percentage of total activity in deposit
$2{\cdot}5\times10^9$	1/2100	—	—	42
$2{\cdot}5\times10^9$	1/2100	1/224	—	19
$2{\cdot}5\times10^9$	1/2100	—	1×10^9	61
$2{\cdot}5\times10^9$	1/2100	1/224	1×10^9	90
$3{\cdot}5\times10^9$	—	1/128	1×10^9	3
$2{\cdot}5\times10^8$	1/1200	—	—	<1
$2{\cdot}5\times10^8$	1/1200	1/224	—	<1
$2{\cdot}5\times10^8$	1/1200	—	1×10^8	10
$2{\cdot}5\times10^8$	1/1200	1/224	1×10^8	93

* Final dilution.

As can be seen from the Table, at a final concentration of $2{\cdot}5 \times 10^8$ phage particles per ml. in the absence of complement and in the presence of antibody at a concentration which would give I-A, 10 per cent and under of the phage was deposited.

Both antibody and complement were necessary at the higher and at the lower concentration of phage shown in order to bring down 90 per cent of the phage. This experiment was repeated using different complements, human and guinea-pig, and different batches of red cells and gave similar results each time.

Rate of I-A reaction measured with labelled phage

The rate at which the pre-formed phage-antibody-complement complex was adsorbed to red cells was measured by incubating 1 ml. of labelled T2 suspension at 4×10^9/ml., 0·8 ml. of 1/300 antiserum, 0·4 ml. of 1/16 complement and 1·4 ml. of diluent for 1 hr. at 37°. Of 50 per cent red cells 0·4 ml. was then added, and samples removed from the mixture at intervals, pipetted into an ice-cold tube and centrifuged to stop the reaction. The distribution of radio-activity in supernate and pellet was determined. This reaction happened very quickly ; 50 per cent of the phage was adherent in 4 min. and the reaction was complete in 25 min. When the rate of union between phage, antibody, complement and red cells was measured from the time of mixing of all four reagents to complete immune-adherence, a much longer time was required, *i.e.*, 18 min. for 50 per cent adherence and 55 min. for 100 per cent adherence. Presumably the time difference was required for the formation of the phage-antibody-complement complex. From neutralization experiments it was found that 95 per cent of the phage was inactivated in 3 min., so that we conclude that the slowest part of the I-A reaction must be the fixation of complement.

The results of these experiments are plotted in Fig. 1 and 2. Also plotted in Fig. 2 is the course of inactivation of the phage in the complete reaction mixture.

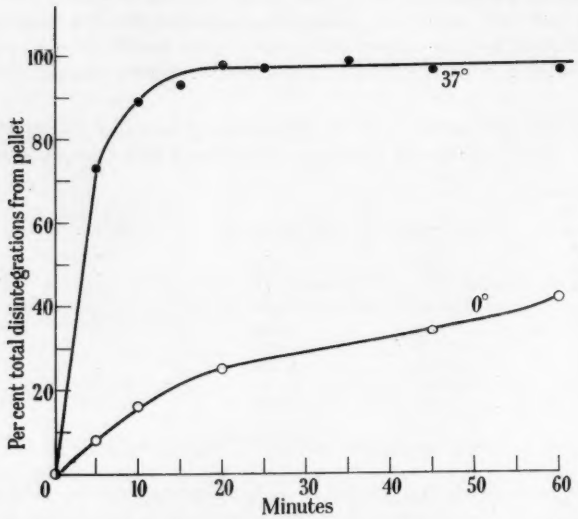

FIG. 1.—The rate of adherence of pre-formed phage-antibody-complement complex to red cells at 37° and at 0°.

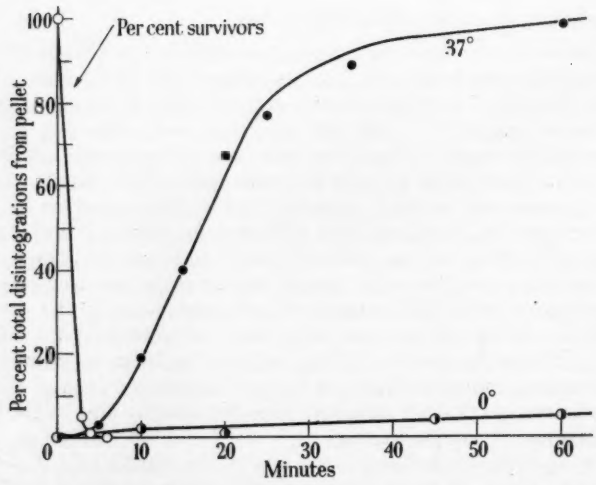

FIG. 2.—The rate of union of phage, antibody, complement and red cells at 37° and at 0°. Also shown is the rate of inactivation of T2.

Measurements of rate of reaction and rate of adherence were repeated in the same way but using ice-cold reagents and keeping the reaction mixtures, one of which had been previously incubated for 1 hr. at 37° before addition of red cells, in an ice-bath. Negligible fixation of complement occurred at 0°, which is consistent with the fact that the third component of complement, C'3, is only fixed at 37° (Mayer, Levine, Rapp and Marrucci, 1954). It also appears that the rate of adherence, as opposed to the rate of reaction, is very much slower at 0°, perhaps because of the reduced rate of collision at the lower temperature.

The rates of reaction obtained with the bacteriophage system are similar to those obtained by Nelson with *Staphylococcus aureus* as the antigen.

DISCUSSION

Immune-adherence has been described for a number of different antigen systems. Nelson has described the reaction with bacteria, with starch and zymosan particles, with rickettsiae and with a large virus, vaccinia. Turk (to be published) has demonstrated that soluble polysaccharide antigens from a number of bacteria and soluble protein antigens undergo immune-adherence. The different nature of these antigens emphasizes that immune-adherence is a general immunological phenomenon.

The haemagglutination technique (Nelson and Woodworth, to be published) for measuring immune-adherence appears to be a valuable one for investigating the behaviour in this reaction of small antigens which cannot be counted under the microscope. It was important therefore to check with one small antigen system that immune-adherence could be demonstrated by another technique as well as by haemagglutination. In the two kinds of experiment just described, although close quantitative comparison is difficult, it would appear that the general proportions in which the reagents acted in haemagglutination and in labelled phage experiments are of the same order. The results suggest that haemagglutination was caused by adherent sensitized phage particles. It can be concluded that with small, as with large, particulate antigens the haemagglutination technique is a valid one for investigating immune-adherence.

Finally, it should be stressed that in experiments in which complement is used in high concentrations, particular attention must be paid to the natural antibodies it contains.

SUMMARY

Bacteriophage T2, in the presence of anti-phage serum and complement, agglutinates human red cells by immune-adherence. Both phage and antiserum can thus be titrated by a haemagglutination pattern test.

Phage T2 in the presence of complement alone will agglutinate red cells. Absorption of complement with *Bact. coli* prevents haemagglutination in the absence of antiserum if complement is used at an initial dilution greater than 1/4.

Experiments with phage labelled with ^{35}S suggest that both normal guinea-pig and human sera at high concentration contain small amounts of phage antibody.

In the presence of immune serum and complement over 90 per cent of the phage particles adhered to human red cells at 37°.

The rate of adherence of the pre-incubated phage-antibody-complement complex to the red cells and the rate of union of all four reagents from the time

of mixing were measured at 37° and 0°. Fixation of complement appears to be the slowest part of the reaction.

It is concluded from a comparison between haemagglutination experiments and experiments with phage labelled with ^{35}S that haemagglutination is probably caused by the adherence of the phage particle itself to the surface of the red cell and that the haemagglutination technique is therefore a valid one for the investigation of immune-adherence with small antigens.

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THE HETEROGENEITY OF INFECTIVE VIRUS PARTICLES WITHIN A SINGLE INFLUENZA VIRUS STRAIN

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THE separation of influenza virus preparations into rapidly and slowly eluting fractions, by a method of fractional elution from red cells, has recently been described (Smith and Cohen, 1956). Preliminary investigation of some of the properties of the two fractions was also reported. They were shown to be identical in infectivity and enzymic activity against a soluble substrate, but to differ in their toxicity for mice intracerebrally.

The difference in enzymic activity against red cell receptor substrate suggested that the fractions might also differ in their reactions with related substances. Inhibition of haemagglutination can be effected by two types of inhibitors present in normal sera and by the specific antibodies in immune sera but, of these, only the inhibitor of Francis (1947) resembles red cell receptor substrate in being destroyed by either virus enzyme or by the receptor-destroying enzyme (RDE) of *Vibrio cholerae* (Anderson, 1948). This paper reports an investigation of the behaviour of the rapidly eluting (RE) and non-eluting (NE) components of standard virus preparations in serological reactions with normal and immune rabbit sera. The results indicate that the heterogeneity of the fully infective virus particles is due to differences more fundamental than simple quantitative differences in the amounts of enzyme carried per particle.

MATERIALS AND METHODS

Viruses

The strain used for virus fractionations was the Influenza A prime strain Ash, isolated in this laboratory in 1953 and subsequently maintained by allantoic passage in embryonated hens' eggs.

In addition the following standard reference strains were used: PR8 (type A); FM1 (A prime); A/Eng/1/51 (A prime) and Lee (type B). These have all been maintained for several years by serial egg passage.

Allantoic fluid seeds of the viruses were stored at − 70°. Batches of virus were prepared as required by inoculation of 11-day embryonated hens' eggs by the allantoic route. The standard inoculum was 0·2 ml. of seed virus diluted 10^{-3} or 10^{-4} in nutrient broth containing penicillin 2500 units/ml. and streptomycin 3000 μg./ml. After incubation at 35° for 48 hr., the eggs were chilled overnight at 4°, and the infected allantoic fluids were harvested, pooled and stored at 4° until required.

Receptor-destroying enzyme (RDE)

Receptor-destroying enzyme was obtained from agar plate cultures of *V. cholerae* by the method of Burnet and Stone (1947).

Human red cell suspensions

Human group O red cells were obtained from volunteer donors. After separation from the plasma the cells were washed three times in physiological saline and stored at 4° for a period not longer than 7 days. Suspensions of 0·25 or 0·5 per cent in physiological saline were made up as required and standardized by means of a photo-electric densitometer.

Normal rabbit sera

Aliquots of sera obtained from individual rabbits were treated in two ways. One aliquot was inactivated at 56° for 20 min. The other was treated with RDE in order to destroy the α inhibitor. Enough crude RDE was added to give a 1/10 dilution. The mixture was incubated at 37° for 5½–6 hr. and then inactivated at 56° for 20 min. In the early experiments sodium citrate was added to the RDE-treated serum before inactivation but was later found to be unnecessary. Titres obtained with RDE-treated sera were adjusted to correct for the dilution factor.

Immune rabbit sera

Specific antisera were prepared in rabbits by intravenous injection of about 100 agglutinating doses (AD) of virus followed 3 weeks later by a second intravenous injection of 500–1000 AD. Rabbits were bled 6 and 12 days after the second injection. Except where otherwise stated, antisera were inactivated at 56° for 30 min. and then treated with trypsin to destroy non-specific inhibitors of both α and β types. Crystalline trypsin (Armour brand) was added to a concentration of 2 mg./per ml.

Egg white

Crude egg white obtained from infertile hens' eggs was diluted 1/10 in phosphate buffer saline at pH 7.

Preparation of virus fractions

Virus fractions were prepared from 5th or 6th allantoic passes of the strain Ash. Equal volumes of infected allantoic fluid and 0·25 per cent human red cell suspension were chilled, mixed and held at 4° for 30 min. After centrifugation at 2000 r.p.m. for 3 min. the supernatant was discarded. The cells were re-suspended in physiological saline at 37°, the volume being one-quarter the original volume of allantoic fluid. After 10 min. virus which had eluted was recovered by centrifugation, the supernatant fluid representing the rapidly eluting fraction referred to hereafter as RE. The cell deposit was again re-suspended in the same volume of physiological saline at 37° and elution allowed to proceed for a further 30 min. Virus eluted within this period was collected by centrifugation and discarded. The cell deposit was then re-suspended in crude RDE, diluted 1/10 in physiological saline and held at 37° for a further 30 min. Virus released from the cells within this period was separated by a further centrifugation. It consisted of some virus which might have eluted slowly in the absence of RDE but chiefly of virus incapable of spontaneous elution. This fraction is therefore referred to as non-eluting (NE) virus.

Except in the first experiment the RE fraction as soon as collected was held at 37° for 30 min., enough crude RDE was then added to give a final dilution of RDE of 1/10 and the mixture held at 37° for a further 30 min. A sample of the original allantoic fluid was treated in the same way except that it was held at 37° for 40 min. before addition of the RDE. In this way the original allantoic fluid and the RE and NE fractions were subjected as nearly as possible to the same physical conditions. The preparations were freed from RDE by centrifugation in a Spinco centrifuge (50 min. with mean RCF 19,000 g), the virus deposits being taken up in volumes of borate buffer saline to give concentration factors of approximately × 50.

Elution rates

The elution rates of the original allantoic fluid and its two fractions were compared by the method described by Smith and Cohen (1956).

Serological tests

Haemagglutination and haemagglutination-inhibition titrations were done by the photo-electric densitometer method of Hirst and Pickels (1942) as modified by Smith and Westwood

(1950) and Belyavin, Westwood, Please and Smith (1951). Titres were obtained from densitometer readings by the use of Lim's nomogram for 50 per cent end-point interpolations (Lim, 1954). Fowl red cells were used for all titrations.

Complement fixation tests were done by the micro-method of Belyavin (1953) in which results are expressed as total fixation areas produced by chess board titrations.

Flocculation tests were done as described by Belyavin (1955).

EXPERIMENTAL

Sensitivity of Virus Fractions to Non-specific Haemagglutination Inhibitors

Normal rabbit sera contain two distinct types of haemagglutination inhibitors designated α and β by Smith, Westwood and Belyavin (1951). The α inhibitor corresponds to that described by Francis (1947) and is often referred to as Francis inhibitor. It inhibits, to high titre, haemagglutination by most type A and type B influenza virus strains which have been heated at 56°/30 min.; it is relatively thermostable and is destroyed by either RDE or trypsin. The β inhibitor inhibits haemagglutination by most A prime strains, whether living or heat-inactivated, and the titres against living virus are usually as high, or even higher, than those against heated virus. It is relatively thermolabile, is not destroyed by RDE but is rapidly destroyed by trypsin. Non-specific inhibitors of the β type have previously been described by McCrea (1946, 1948), Burnet and McCrea (1946) and Chu (1951). The inhibitor of egg white closely resembles the α serum inhibitor being thermostable and sensitive to RDE but the two are certainly not identical.

The sensitivities of Ash allantoic fluid virus and its RE and NE fractions to these various non-specific haemagglutination inhibitors were examined in a number of experiments. Both unheated and heated virus preparations were tested against untreated and RDE-treated normal rabbit sera and in some experiments egg white inhibitor was included. The results are summarized in Table I.

TABLE I.—*Haemagglutination Inhibition Titres of Non-specific Inhibitors against Virus Fractions*

Experiment	Inhibitors	Ash virus fractions: AF L	AF H	RE L	RE H	NE L	NE H	Lee virus L	Lee virus H
1	NRS	212	—	240	—	48	—	136	975
	„ (RDE-treated)	113	—	116	—	25	—	36	71
2	NRS	256	326	358	256	78	47	208	384
	„ (RDE-treated)	206	32	129	<16	<16	<16	<16	<16
3	NRS	—	—	215	610	73	91	—	2048
	„ (RDE-treated)	—	—	<16	<16	<16	<16	—	32
	Egg white	—	—	—	3153	—	1024	—	753
4	NRS	163	186	220	206	40	66	115	471
	„ (RDE-treated)	108	76	181	94	25	29	41	55
	Egg white	16	911	20	1126	<32	362	<16	230

NRS = Normal rabbit serum. L = Unheated (living) virus. H = Heated virus. In all Tables AF = Allantoic fluid. RE = Rapidly eluting fraction. NE = Non-eluting fraction.

Lee virus, unheated and heat-inactivated, was included in the tests because it is the classical indicator strain for the assay of α inhibitor. It serves to show

that the different normal rabbit sera used varied widely in their inhibitor content and that the RDE treatment was effective in removing α inhibitor whilst leaving β inhibitor virtually intact.

Strains of A prime of which Ash is a characteristic example are relatively insensitive to α inhibitor but are particularly sensitive to β and egg white inhibitors. Lee virus has converse sensitivities. Hence it is only with sera devoid of β like that used in experiment 3 that the Ash fractions gave a typical Francis effect, namely a marked rise of sensitivity on heat inactivation. With the untreated sera of the other three experiments titres against living Ash virus fractions were of the same order as titres against the corresponding heated viruses because of the presence of β inhibitor, and with RDE destruction of the α component the greater sensitivity of virus in the living state to β inhibitor became manifest.

In all the experiments the RE and NE fractions exhibited a very striking difference of sensitivity to both the α and β serum inhibitors and also to egg white. Quite consistently RE virus was very much more sensitive. Indeed NE virus has such low sensitivity that it is virtually useless in detecting the presence of β even when this is present in large amount as is shown in experiment 2. The much greater sensitivity of RE virus to α is surprising because α is rapidly destroyed by the virus enzyme. The significance of this anomaly is discussed later. As was to be expected the parent allantoic fluids containing both types of virus particle had an intermediate grade of sensitivity.

Sensitivity of Virus Fractions to Ash Antisera

The reactivities of RE and NE fractions with antisera obtained by immunizing rabbits with Ash virus were compared in both haemagglutination inhibition (HI) and complement fixation (CF) tests. The parent allantoic fluids were also included. Consistent results were obtained with different virus batches and with two different immune sera, one of high and one of low titre. Four experiments are summarized in Table II ; in two of them the same reagents were used for both HI and CF tests.

TABLE II.—*Haemagglutination Inhibition Titres and Complement Fixation Potencies of Ash Antisera against Virus Fractions*

Experiment	Test	AF	RE	NE
1	HA inhibition	—	5,509	1,812
2	„	2,880	5,520	668
3	„	4,400	3,921	1,310
	Complement fixation	14 (479)	14 (477)	13 (543)
4	HA inhibition	11,456	20,480	2,720
	Complement fixation	32 (103)	28 (80)	30 (64)

CF figures give fixation areas in chess board titrations. Figures in brackets give HA titres of the antigens used.

The relative susceptibilities of the virus fractions to haemagglutination inhibition by specific antibodies reflect their behaviour with non-specific inhibitors, the enzymically active RE virus being again very much more sensitive. The CF results present a striking contrast, for within the limits of error of the test equal HA doses of the fractions fixed equal amounts of complement. The

presumption is that they combined with equal amounts of specific antibody. Irrespective of whether the HI and CF antibodies in an immune serum are the same or different, the results show that the haemagglutinin receptors of RE virus are more effectively blocked by a given amount of antibody than are the receptors of an equal AD of NE virus.

Exclusion of Laboratory Contamination of Ash

The possibility that Ash might have become contaminated with another laboratory strain was eliminated by the cross HI tests recorded in Table III. All the viruses in use during the period of investigation were included. There is no indication of any abnormally close relationship of either of the Ash fractions with one of the heterologous viruses. The results do show however that the relative insensitivity of NE virus is operative against the heterologous sera of the antigenically related A prime strains FMI and A/Eng/1/51. Thus, so far as our tests have gone, NE virus has shown consistently lower sensitivity than RE virus to haemagglutination inhibition by all types of inhibitor whether specific or non-specific.

TABLE III.—*Haemagglutination Inhibition Titres of Antisera prepared against Homologous and Heterologous Strains*

Antigens		Antisera: Ash	FM1	A/Eng/1/51	PR8	Lee
Ash	AF	11,456	448	1,036	<200	<200
	RE	20,480	600	1,768	<200	<200
	NE	2,720	<200	258	<200	<200
FM1		5,408	7,360	2,416	800	<200
A/Eng/1/51		4,992	1,280	2,432	<200	<200
PR8		2,008	4,384	<200	9,156	<200
Lee		<200	<200	<200	<200	888

Antigenic Homogeneity of RE and NE Virus

In view of the great difference between the sensitivities of the two fractions to type-specific antibodies it was necessary to consider the possibility that a spontaneous mutation, arising during serial passage of Ash, had resulted in allantoic fluid preparations consisting of a mixture of two antigenically distinct virus types.

Three rabbits were immunized respectively with allantoic fluid virus and its RE and NE fractions. In order to increase the chances of detection of any minor antigenic component peculiar to one or other fraction two bleeds were taken at 6 and 12 days after the second immunizing dose. All the sera were trypsinized and heated at 56°/20 min. to remove non-specific inhibitors. They were also absorbed with fowl red cells overnight at 4° to remove any fowl cell agglutinins. Full cross haemagglutination inhibition tests were done, the results of which are given in Table IV. The pre-immunization sera are not included in the Table as they were all shown to be completely devoid of antibodies.

Individual variability of immunogenic response resulted in sera of different potencies but the results give no evidence of any antigenic differences between the fractions. Incidentally they fully confirm the difference between RE and

TABLE IV.—*Haemagglutination Inhibition Tests with Ash Fractions and Their Corresponding Antisera*

		Antisera against fractions :					
		AF		RE		NE	
Antigens.	Bleed :	1st	2nd	1st	2nd	1st	2nd
AF	.	4,050	3,065	2,500	1,810	6,750	11,059
RE	.	8,750	4,100	5,820	2,980	18,250	14,950
NE	.	758	424	424	<320	928	1,575

NE fractions in their sensitivities to haemagglutination inhibition by type specific antibodies. Allantoic fluid again fell into an intermediate position. The fact that the immunogenic potencies of allantoic fluid, RE and NE fractions followed a reverse order so that the NE antisera had much higher titres than the RE antisera was probably due to chance but might possibly represent another important difference between the fractions and calls for further investigation.

Flocculability of RE and NE Fractions

The direct flocculation of influenza viruses by specific antisera, described by Belyavin (1955) is particularly suited for the antigenic analysis of strains because it is independent of most of the external variables which complicate the haemagglutination inhibition and complement fixation tests. Flocculation experiments were done with the Ash fractions and the specific rabbit antisera prepared against them. The results were surprising for it was expected that the low sensitivity of NE virus to haemagglutination inhibition by specific antibodies would be reflected in its flocculation behaviour. Titrations in which the antigens were used in constant dilution, after equation on the basis of haemagglutination titres, against falling dilutions of antisera showed that quite as high titres were obtained with NE virus as with RE virus ; moreover the reaction occurred more rapidly with NE virus and the resulting precipitates were bulkier. The most obvious conclusion is that NE virus combines with more flocculating antibody than does RE virus per haemagglutination dose. But the complement fixation tests had indicated that amounts of antibody absorbed per HA dose were equal. Any significant differences between amounts of flocculating antigen in the fractions should be reflected by their dilution end-points against a constant amount of antibody. Cross titrations were therefore done with constant antiserum dilutions against falling dilutions of the antigens. The results are tabulated in Table V

TABLE V.—*Haemagglutination Inhibition and Flocculation Tests with Ash Fractions and Their Corresponding Antisera*

			HI titres of antisera			Flocculation titres of antigens against 1/20 dilutions of sera		
Experiment	Antigens		AF	RE	NE	AF	RE	NE
1								
Concentrated fractions	RE (142)*	.	4,200	3,310	7,200	3	3	4
	NE (102)	.	1,690	922	2,740	8	8	16
2								
Unconcentrated fractions	RE (40)	.	6,400	4,660	14,182	<2	2	2
	NE (35)	.	1,750	1,235	3,600	4	6	8

* Bracketed figures give HA titres of fractions after attempted equation.

and the haemagglutination inhibition titres of the sera are included for comparison. The earlier tests were done with virus concentrates prepared by centrifugation of the various fractions and the unexpected results raised the suspicion that with NE virus such manipulation might have resulted in preferential destruction of haemagglutinin receptors. The later tests with unconcentrated fractions refuted this possibility. Quite consistently the NE virus proved to be the more potent flocculating antigen.

Is NE Virus a "Q" Variant?

Van der Veen and Mulder (1950) described a phase variation in influenza viruses which they designated P-Q-R variation. Among closely related virus strains they found some which reacted typically to high titre with homologous ferret antiserum and to much lower titre with heterologous antisera. These they called P variants. Others, the R variants, reacted to high titre with both homologous and heterologous antisera. The Q variants gave very low titres with both. Isaacs and Andrewes (1951) showed that P-Q variation may occur in a single virus strain and were able to induce the transformation in either direction by experimental manipulation.

The cross titrations summarized in Table IV show that the serological reactivity of the NE fraction of Ash virus resembled that of a Q variant. This raised the suspicion that the heterogeneity of Ash allantoic fluids might represent a mixture of virus particles, some being in the Q phase and if so that loss of elution efficiency might be an essential feature of Q variation.

A strain of virus A/Italy 420/56 known to be in the Q phase was obtained through the courtesy of Dr. Isaacs of the World Influenza Centre, London. In haemagglutination inhibition tests with normal rabbit sera it showed the same low sensitivity as NE/Ash fraction to the non-specific serum inhibitors. When tested for elution efficiency however it behaved quite differently. The comparative experiment recorded in Table VI shows that quite high enzymic potency can be retained by virus in the Q phase. Indeed elution of A/Italy 420/56, although a little slower initially than that of unfractionated Ash, gave higher yields at 40 and 60 min. The results also strikingly illustrate the difference in elution behaviour of the RE and NE fractions. Thus, although it is possible that NE/Ash particles are in the Q phase this type of variation does not provide a full explanation of the heterogeneity of Ash preparations.

TABLE VI.—*Comparison of Elution Rates of Ash Fractions and the Q Variant of A Italy*/420/56

Time (min.)	AF	RE	NE	A/Italy 420/56
10	14	38	3	6
20	28	55	6	19
40	35	75	10	54
60	36	64	13	52

Figures are the percentages of adsorbed virus eluted.

Electron Microscopy of RE and NE Fractions

The fact that Ash virus is a partially filamentous strain raised the possibility that elution fractionation results in the separation of filaments from spheres and that different reactivities of RE and NE fractions are correlated with these

distinctive morphological types. It has not been possible to investigate this thoroughly but a few electron micrographs obtained by courtesy of Dr. Valentine of the National Institute for Medical Research gave no support to this suggestion. They showed the presence of both spheres and filaments in about the same proportion in RE and NE fractions. It is possible that many filaments may have been disrupted during the manipulations of elution and centrifugation or by the freezing and thawing involved in storage of virus preparations at −70°, but since electron micrographs of both concentrated and unconcentrated fractions showed no difference in the relative proportions of the two morphological forms it seems unlikely that fractionation by elution results in their segregation.

DISCUSSION

In a previous publication Smith and Cohen (1956) showed that influenza virus, synthesized as a result of egg infection, consists of enzymically heterogeneous virus particles and that the enzymically inactive component is not merely incomplete or degraded virus because its infectivity is equal to that of the active component. The present investigation affords evidence that the heterogeneity cannot be fully accounted for by simple quantitative differences in the amounts of enzyme carried per particle but must represent more fundamental differences of particle constitution or structure.

In addition to their different enzymic activities the RE and NE fractions have different affinities for, and susceptibilities to, both haemagglutination inhibitors of normal sera and the type specific antibodies of immune sera ; yet there are no apparent qualitative differences of antigenic constitution. It is possible that the virus particles of an infective fluid vary quantitatively as distinct from qualitatively in their antigenic make-up. It is not known how many different antigens and their corresponding antibodies are concerned in the various types of serological reaction but there is good evidence that flocculation and haemagglutinin receptors occupy different sites on the virus surface (Belyavin, 1956) and Smith, Belyavin and Sheffield (1955) have shown that more than one virus antigen may be concerned in the complement fixation reaction. The virus serum mixtures are therefore antigenically complex and differences in the relative proportions of various antigens in RE and NE fractions might well result in the greater susceptibility of RE virus to haemagglutinin inhibition and its lesser sensitivity to flocculation. There is however no evidence to show in which fraction or in which antigenic component deficiency lies. Equation of the fractions on the basis of HA titre does not ensure that the test suspensions contain either equal numbers of virus particles or equal amounts of virus material. They might equally well contain different numbers of virus particles provided that the fractionation had resulted in a partial segregation of those endowed with many haemagglutinin receptors from those endowed with relatively few. The complement fixation titres do in fact suggest that our fractions after HA equation contained equal amounts of virus but from the results of the flocculation titrations a natural assumption is that the NE suspensions contained more flocculating antigen and hence presumably more virus than the RE suspensions. In order to determine the relative proportions of reactive constituents in the virus particles themselves, as distinct from the proportions in suspensions of equal HA titre, equation on the basis of particle counts by electron microscopy would be required.

Unfortunately means for extending the investigation in this direction were not available.

The different activities of the fractions may depend on variations other than quantitative antigenic variations. Smith and Westwood (1950) demonstrated the fallacy of the common assumption that the Francis phenomenon is due to destruction of enzyme during conversion of virus to the indicator state by heat inactivation. They adduced cogent evidence that the greater susceptibility of indicator virus to inhibitor is due to distortion of the haemagglutinin receptors so that a given amount of adsorbed inhibitor has much greater inhibitory effect. This offers some support to the hypothesis that the heterogeneity of infective allantoic fluid virus likewise depends largely on variations of configuration of surface receptors. It is significant that the enzymically active RE fractions showed much greater sensitivity to Francis inhibitor of normal sera although its enzyme is capable of destroying this inhibitor. The hypothesis does not exclude the possibility of associated quantitative antigenic differences although these are not necessary to explain the anomalies of behaviour. The occlusion, total or partial, of one or other type of receptor by steric hindrance would determine relative degrees of activity in different serological tests and could incidentally lead to a totally erroneous estimate of the number of virus particles present, or the amount of receptor substance per particle, from titration results.

It is not known whether the heterogeneity described represents the presence of only two distinct types of virus particle or a continuous series ranging from enzymically highly active to totally inactive. The elution curves suggest that the latter alternative is the more probable but definite evidence is lacking. The question cannot be settled by this type of approach because fractionation by elution is incapable of yielding enzymically homogeneous components. Active particles can release adjacent inactive virus from the red cells and inactive virus can prevent spontaneous elution of some active particles by occlusion and can also be contaminated with such particles from re-adsorption. The elution fractionation therefore merely alters the relative proportions of active and inactive particles in the eluates obtained. Theoretically pure preparations of the non-eluting virus should be obtainable by several successive fractionation cycles but the heavy losses of virus entailed at each cycle make it a practical impossibility. The proportion of active virus in our NE fractions however must have been extremely small.

It is improbable, though not impossible, that the different types of virus particle arise by spontaneous mutation. It is true that Smith, Westwood, Westwood and Belyavin (1951) described a mutant strain of PR8 virus which resembled NE fraction virus in several respects. It differed from its parent strain in having less sensitivity to the Francis inhibitor of normal serum and to haemagglutination inhibition by both homologous and heterologous immune sera whilst retaining equal complement fixation potency. Unlike NE virus however it was enzymically active against red cell substrate. The Q variant used in the present investigation showed similar characteristics. In a previous paper however we reported complete failure to obtain any evidence that RE and NE virus fractions consist predominantly of different genetically stable substrains of Ash. Each fraction was passaged serially at limit dilutions but all eggs which became infected, without any exception, yielded allantoic fluids containing a mixture of RE and NE virus particles. There was no suggestion even that their

relative proportions were any different from those in the standard Ash preparations (Smith and Cohen, 1956).

The virus in infective allantoic fluids represents the product from several cycles of replication throughout which the synthesis of various components must continue although not necessarily at a uniform rate. Moreover particles formed late in the replication period have a relatively short time for any maturation which may occur. Under such conditions it would be surprising if all particles were perfectly uniform in constitution and structural configuration. Much more likely is the production of particles having slight variations of surface pattern and endowed therefore with different degrees of serological reactivity. The production of "incomplete" virus under certain conditions which can be deliberately induced is in line with this hypothesis. To establish its validity comparative tests of fractions, equated on the basis of particle counts by means of electron microscopy, are needed. Investigations on these lines should yield further insight into the mechanisms of virus replication.

SUMMARY

Rapidly eluting (RE) and non-eluting (NE) influenza virus fractions differ in several ways. The RE virus in spite of its greater enzymic activity is much more sensitive to the non-specific haemagglutination inhibitors of both α and β type present in normal rabbit sera and to egg white inhibitor. It is also much more sensitive to HA inhibition by the antibodies of specific immune sera.

Complement fixation and flocculation tests with immune sera gave anomalous results. The two fractions, after equation on the basis of HA titres, showed equal CF activities but in flocculation tests NE virus gave higher titres and formed more abundant precipitates than RE virus.

Preliminary examination by electron microscopy showed no significant difference in the proportions of spheres and filaments in the two fractions.

Antigenic analysis failed to reveal any antigenic differences between the fractions.

Alternative hypotheses to account for these differences in behaviour are discussed and it is suggested that the virus particles in infected allantoic fluids differ in their surface configuration.

Thanks are due to Dr. R. Valentine for the examinations of virus fractions by electron microscopy. Expenses of the work were partially defrayed from a Medical Research Council grant.

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ACTIONS OF ADENOVIRUS TYPES 5 AND 7 IN CULTURES OF RABBIT KIDNEY TISSUE

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THE cytopathic effect of adenoviruses in cultures of rabbit tissues has been reported by several workers. Rowe, Huebner, Gilmore, Parrott and Ward (1953) first described a cytopathic action of these agents in cultures of suckling rabbit kidney and trachea. The same group of workers (Rowe, Huebner, Hartley, Ward and Parrott, 1955) demonstrated the serial propagation of adenovirus types 1 and 2 in cultures of rabbit trachea and further noticed that some types, especially types 3 and 4, caused a cytopathic effect of limited degree, usually transient and not reproducible in serial passages. Balducci, Zaiman and Tyrrell (1956) used cultures of trypsinized rabbit kidney for serum neutralization tests with adenoviruses types 1 to 6, observing also a limited cytopathic effect with production of little infective virus. These results suggest that the cytopathic effect of adenoviruses in rabbit kidney cultures may be unrelated to production of infective virus as in the case of influenza virus in HeLa cells described by Henle, Girardi and Henle (1955). The toxic nature of the cytopathic effect of adenoviruses has been suggested by Harford, Hamlin, Parker and van Ravenswaay (1956). This suggestion is supported by our results (Pereira and Kelly, 1957) on the study of dose-response curves of adenovirus titrations in HeLa cells where early and late cytopathic effects are distinguished and attributed respectively to toxic and infective activities of the virus. The dissociation between cytopathic effect and virus multiplication is obscured in the work mentioned by the high susceptibility of HeLa cells to these viruses. In the present study such a dissociation is more clearly demonstrated by using the less susceptible rabbit kidney cells. Both adenovirus types 5 and 7 are shown to exert typical cytopathic effect in rabbit kidney cultures but evidence of virus multiplication could only be obtained with adenovirus type 5.

MATERIALS AND METHODS

Tissue cultures

Rabbit kidneys were obtained from 10-day- to 4-week-old animals and grown in plasma clot roller tube cultures as described by Robbins, Weller and Enders (1952). The nutrient fluid consisted of bovine amniotic fluid with 5 per cent normal horse serum, 0·25 per cent lactalbumin hydrolysate (Nutritional Biochemical Corporation, Cleveland, Ohio), 0·01 per cent soya bean trypsin inhibitor, 0·001 per cent phenol red, penicillin (100 units/ml.) and streptomycin (0·1 mg./ml.). Each tube contained 5 explants and 1 ml. of medium. After 4–6 days' incubation at 37° most explants were surrounded by an outgrowth consisting mainly of epithelial cells. The fluid was then removed and each tube received 0·9 ml. of fresh medium followed by 0·1 ml. of the virus inoculum appropriately diluted in broth saline

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(10 per cent tryptic digest broth in normal saline). The tubes were re-incubated at 37° and examined microscopically for evidence of cytopathic effect at suitable intervals during the following 7 days.

HeLa cells were propagated and used for virus titrations by methods described elsewhere (Pereira and Kelly, 1957).

Virus strains

The prototype strains of adenovirus type 5 and type 7 were received from Dr. R. J. Huebner, National Institutes of Health, Bethesda, Maryland. The virus materials used in the present work consisted of the 5th and 2nd HeLa cell passages in our laboratory of types 5 and 7 respectively. The type 5 preparation had an infectivity titre for HeLa cells of $10^{8.5}$ TCD_{50} per ml. and a complement fixation titre of 320 against human convalescent serum. The corresponding titres of the type 7 preparation were $10^{6.8}$ TCD_{50} per ml. and 160.

Growth curves

Each of 14 tubes of rabbit kidney cultures were inoculated with a given virus dose. At daily intervals the contents of 2 tubes were pooled, crushed in a TenBroeck tissue crusher, centrifuged at about 2000 r.p.m. for 10 min. and the supernatant stored at − 15°. The original virus material and each of the daily samples were titrated in the same batch of HeLa cell cultures.

Inactivation curves

An appropriate dilution of each virus was made in the nutrient fluid used for rabbit kidney cultures and distributed in 1 ml. volumes. One sample was immediately stored at − 15° and the rest were incubated at 37°. At daily intervals samples were transferred to − 15°. All samples were titrated in the same batch of HeLa cell cultures.

Virus titrations

Serial dilutions of the virus materials were made in broth saline in 10-fold steps, and 0·1 ml. volumes of each dilution were inoculated into each of 4 tubes of HeLa cells containing 0·9 ml. of medium. The tubes were incubated for 8–13 days, with a fluid change at 6 days. All titration end-points of each experiment were read on the same day and related to the titre of the original virus inoculum. End-points were calculated by the method of Reed and Muench (1938) and the titres given represent the number of 50 per cent tissue culture doses (TCD_{50}) per ml.

Complement fixation tests were performed in Perspex trays using 0·1 unit volumes, 2 units of complement and overnight fixation at 4°. Details of the technique have been published (Pereira, 1956). The tissue culture materials were heated at 60° for 15 min. and tested in 2-fold dilutions starting at $\frac{1}{2}$ against a pool of human convalescent sera.

RESULTS

Both virus strains under study caused marked cytopathic effect when inoculated in large doses into rabbit kidney cultures. The intensity and the time of onset of this effect varied with size of virus inoculum and the doses required to cause cytopathic effect in 3 and 6 days were of the order of $10^{5.0}$ and $10^{4.5}$ HeLa cell TCD_{50} respectively. The cytopathic effect of each of the two virus types corresponded closely to the effects observed in rabbit tissue cultures by Rowe *et al.* (1955) and Balducci *et al.* (1956) and the non-progressive character of the effect caused by small virus doses was a typical feature.

Evidence for the multiplication of type 5 virus in rabbit kidney cultures was provided by its propagation through 7 passages in these cultures with in all a 10^8-fold increase in infectivity and with reproduction of cytopathic effect at each passage. Virus multiplication is also suggested by the results of growth curves

shown in Fig. 1 in which we also include the inactivation curve of adenovirus type 5 at 37° in the medium used for rabbit kidney cultures. In both growth curves, infectivity levels remained approximately constant. This is contrasted with a regular daily drop of about $10^{0.25}$ TCD_{50} in the inactivation curve. The

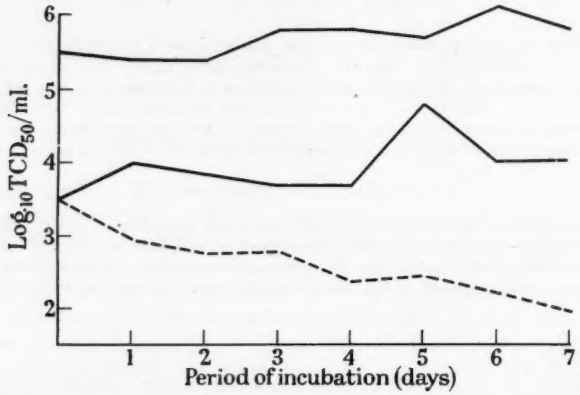

FIG. 1.—Infectivity levels of adenovirus type 5 in rabbit kidney cultures (continuous lines) and in cell-free medium (broken line).

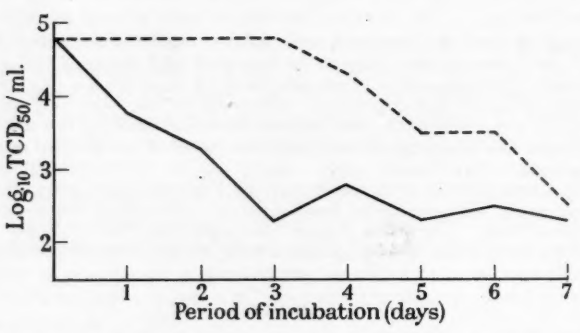

FIG. 2.—Infectivity titres of adenovirus type 7 in rabbit kidney cultures (continuous line) and in cell-free medium (broken line).

series of cultures inoculated with $10^{3.5}$ TCD_{50} showed no evidence of cytopathic effect throughout the period of observation whereas in the cultures inoculated with $10^{5.5}$ TCD_{50} a widespread cytopathic effect was observed from the 3rd day of incubation. No complement-fixing antigen could be demonstrated in any of the samples from the cultures inoculated with the smaller virus dose. With the larger inoculum, however, complement-fixing titres rising from 2 at the first day to 12 at the end of the experiment were detected.

The result of a similar experiment with adenovirus type 7 is shown in Fig. 2. In contrast with type 5, infectivity titres decreased progressively during incuba-

tion and were lower than those of the corresponding virus inactivation curve. Typical cytopathic effect was observed from the 4th day of incubation. No complement-fixing antigen could be detected in any of the samples obtained. Repeated attempts to propagate adenovirus type 7 through serial passages in rabbit kidney cultures by the technique described were unsuccessful and no cytopathic effect was observed except in first passages.

DISCUSSION

The present results provide further evidence for the toxic nature of the cytopathic effect of adenoviruses and show that this effect is not necessarily related to virus multiplication. This is particularly clear with adenovirus type 7 which can cause a marked cytopathic effect in the absence of any evidence of virus multiplication. Production of complement-fixing antigen and infective virus showed some correlation in our experiments and no evidence was obtained for the association of cytopathic effect with production of non-infective virus material as observed by Henle *et al.* (1955) with influenza virus in HeLa cells. Failure to demonstrate this association may have been due to the unsuitability of the complement fixation test for revealing non-infective virus materials.

The suggestion of a toxic nature of the early cytopathic effect of adenovirus type 5 in HeLa cells (Pereira and Kelly, 1957) was supported by the study of dose-response curves indicating that this effect is not initiated by the independent action of virus units. A detailed study of dose-response curves was not carried out in the present work but a few titrations of adenovirus type 5 and type 7 in cultures of rabbit kidney with 0·3 log. dilution steps and 8 cultures per dilution gave sharp cytopathic effect end-points, highly suggestive of a dose-response curve with a slope higher than that of the one-particle curve and therefore inconsistent with the hypothesis of independent action of virus units in the initiation of this effect. According to these results, the cytopathic effect of adenoviruses in rabbit kidney cultures might be explained either by the synergic action of virus particles or by the presence of toxic materials in the virus preparations used.

SUMMARY

The cytopathic action of adenoviruses type 5 and type 7 in rabbit kidney cultures was confirmed. Limited multiplication of adenovirus type 5 in these cultures was demonstrated by the results of serial passages and by growth curve experiments. With adenovirus type 7, however, no evidence of virus multiplication could be obtained. It is suggested that the cytopathic effect observed in this system is of a toxic nature, not necessarily associated with virus multiplication.

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EFFECT OF HOMOLOGOUS BONE MARROW INJECTIONS IN X-IRRADIATED RABBITS*

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RECOVERY from otherwise lethal doses of total body X-irradiation in rodents is promoted both by lead shielding of the surgically exteriorized spleen during irradiation (Jacobson, Marks, Gaston, Robson and Zirkle, 1949), and by injection or transplantation of living blood-forming cells after irradiation (Lorenz, Uphoff, Reid and Shelton, 1951). In protection by intravenous injection of bone marrow it has been shown that the introduced cells proliferate and repopulate the host's depleted haemopoietic and lymphopoietic tissues (Ford, Hamerton, Barnes and Loutit, 1956).

However, whereas spleen shielding in the mouse spectacularly increases survival and markedly hastens the recovery of the blood-forming tissues (Jacobson, 1952), it is less effective in the rat (Jacobson, Simmons, Marks, Robson, Gaston and Bethard, 1950), and minimal effect is observed in the rabbit (Jacobson, Marks and Gaston, 1953). Similarly bone marrow injections in the mouse seem to exert a greater protective activity than does marrow treatment in the rat (Fishler, Cole, Bond and Milne, 1954).

Because the spleen of the mouse is unlike that of many other mammals, in that it continues to produce haemopoietic cells on a large scale in adult life, and because of the relative radio-resistance of the intestinal tract in this species, the possibility arises that the response of the irradiated mouse to spleen and marrow treatment is particularly favourable. Hence the effect on other animals of post-irradiation administration of haemopoietic suspensions is considered to be of interest.

In this report marrow transplantation experiments with rabbits are described in which the sex difference between the heterophil nuclei of male and female is used to provide a biological "marker". It is shown that implanted female marrow cells survive, proliferate and contribute heterophils to the peripheral blood of male irradiated animals for considerable periods of time. The effect on survival of the host is not great however.

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† Joint British Postgraduate Medical Federation Travelling Fellow and George Gorham Peters Fellow.

MATERIALS AND METHODS

Young adult male New Zealand white rabbits, weighing 2120–2990 g. were used as recipient animals.

X-rays were produced by a 250 kV.p. Phillips machine operating at 15 mA. A 0·4 mm. Sn, 0·25 mm. Cu and 1·0 mm. Al filter was used giving a half value layer in copper of the filtered beam of 2·5 mm. The exposure rate was 50 R per min. at 50 cm. centre of animal to target distance, measurement being made in air with a Victoreen ionization chamber.

The marrow for post-irradiation injection was obtained from young adult female New Zealand white rabbits, weighing 2450–3000 g. The donor and recipient animals came from different stocks and would not accept skin homografts one from the other. The donor rabbits were killed by Nembutal intravenously. Using aseptic procedure the marrow from both femurs, tibias and humeruses was removed and suspended gently in ice-cold Earle's buffered saline solution. This suspension was then centrifuged at 600 r.p.m. for 5 min. and the supernatant fat discarded. The residue was made up to 10 ml. by adding further Earle's solution and gently mixing. The number of nucleated cells per cu. mm. was determined by making a dilution with 2 per cent acetic acid in a white cell counting pipette and the cells counted on a standard haemocytometer. Differential counts were made on smears of the suspension. All injections were given within 1 hr. of the death of the donor and were administered intravenously into the marginal ear vein of the recipient rabbit 1–3 hr. following irradiation. The control irradiated rabbits each received 10 ml. of Earle's solution intravenously.

After treatment the rabbits were housed individually in metal cages and were fed Purina rabbit chow, supplemented by hay and carrots, and were given unlimited water.

Total leucocyte counts and differentials, haemoglobins and micro-haematocrits were determined every 2 days for the first 30 days and weekly thereafter until 60 days.

The structural difference between the nuclei of heterophil leucocytes in male and female rabbits (Lüers, 1956) was confirmed and then used as a biological cell marker characteristic of the donor identifiable in the host. In 50 normal female rabbits characteristic nuclear " drum sticks " could be easily recognized. These closely resembled those seen in humans (Davidson and Smith, 1954), each consisting of a well-defined solid chromatin nodule about 1·5 μ in diameter, joined by a single fine chromatin strand to one lobe of the nucleus. The nodules tended to be more round and the chromatin strands to be shorter than those seen in the human. They occurred on the average 1 in every 14 heterophils. With experience small chromatin clubs, minor lobes, sessile nodules and racket structures were found to be easily distinguishable from typical " drum sticks ". In blood films from 50 male rabbits no " drum sticks " were found after examining 300 heterophils.

By injecting marrow from a female rabbit into an irradiated male, survival and proliferation of the donor cells in the host was deduced if heterophils bearing characteristic nuclear " drum sticks " could later be identified in the host's peripheral blood and marrow. Blood films were taken from the ear on alternate days and stained by Wright's method and the heterophils examined. Before accepting that the heterophils were at least in part derived from the transplanted bone marrow a minimum of 6 cells showing these discrete solitary chromatin nodules had to be found.

All animals dying were autopsied. The brain was not examined. A pencil of marrow was dissected from the right femur, the sample always being taken from a comparable portion at the mid-point of the shaft. Most tissues were fixed in formol-saline, but the marrow was fixed in Helly's fluid, and all were routinely stained with haematoxylin and eosin. Special stains were used where necessary, and marrow smears were stained by Wright's method.

The criteria for estimating the effect of homologous bone marrow, were 60-day mortality, 30-day mortality, mean survival time of those dying, weight changes, recovery of the peripheral blood picture with the appearance of " labelled " cells, and histological evidence of bone marrow and lymphoid tissue recovery.

Experimental procedure

In this experiment 4 groups of animals were studied as indicated in Table I. Group I were non-irradiated animals given homologous bone marrow intravenously. Groups II, III and IV were irradiated in pairs, receiving 800 r, 900 r and 1000 r total body x-irradiation respectively. Bone marrow was given to 1 animal from each pair after the irradiation.

TABLE I.—*Effect of Homologous Bone Marrow on Survival of Rabbits Exposed to Total Body X-irradiation*

Group	X-ray dose	Mean number of nucleated marrow cells I–V ($\times 10^6$)	Total number of rabbits	Mortality: 1 day No.	Mortality: 1 day Per cent	Mortality: 30-day No.	Mortality: 30-day Per cent	Mortality: 60-day No.	Mortality: 60-day Per cent	Marrow "takes" No.	Marrow "takes" Per cent	Mean survival time of those dying (days)
I	0	1500	6	0	0	0	0	0	0	0	0	—
II	800 r	0	12	1	8	6	50	6	50	0	0	9
	800 r	1600	12	1	8	6	50	6	50	2	15	11
III	900 r	0	13	3	23	11	83	12	92	0	0	7·7
	900 r	1200	13	2	15	7	54	7	54	5	38	7·0
IV	1000 r	0	16	3	19	16	100	16	100	0	0	8·5
	1000 r	1320	16	3	19	16	100	16	100	10	62	12·3

RESULTS

Survival

All animals in Group I survived indicating no deleterious effect from marrow treatment alone.

In Group II (800 r), 50 per cent of both the marrow-treated and those not treated succumbed within 30 days. The slight prolongation of the mean survival time of those dying amongst the marrow-treated was not significant.

In Group III (900 r), of the 13 animals receiving bone marrow 6 (46 per cent) survived the 60-day period of observation. Among the 13 untreated irradiated controls only 1 (8 per cent) survived to the end of the experiment.

In Group IV (1000 r), all animals died within 22 days. However, the mean survival time of the marrow-treated (12·3 days) was slightly, but significantly, onger than in the corresponding controls (8·5 days).

Weight

Differences between the weight curves of those treated and those not treated with marrow were not marked; but there was a great difference between the weight curves of animals dying and surviving (Fig. 8).

Haematological Studies

Non-irradiated marrow-treated rabbits (Group I)

The intravenous injection of homologous bone marrow had no effect on the normal values of the peripheral blood. No "labelled" cells were seen in smears even in the period immediately following the injection.

Rabbits exposed to irradiation without marrow treatment

Following total body irradiation there was a drastic depression of the leucocyte count which reached its lowest point at the 3rd to the 5th day. In the group given 1000 r the count remained in the range 320–750 cells per cu. mm. until death. In those exposed to 800 r and 900 r however, the count began to increase at the 5th to the 7th day and fell again between the 11th and 12th day—the so-called "abortive rise" (Zirkle, 1954). Recovery from this secondary reduction began about the 14th day and the white cell count continued to rise reaching normal levels at about 21 days. In all animals the mean heterophil value varied

parallel with that of the total leucocytes, but the lymphocytes were slower to recover, barely reaching normal levels at 60 days. Degenerating granulocytes, giant heterophils, metamyelocytes and myelocytes were occasionally seen in the peripheral blood but no cells bearing nuclear " drum sticks ".

A progressive normochromic anaemia, reaching its maximum 14 days after X-irradiation and with recovery by about 30 days, occurred in all animals. During recovery nucleated erythrocytes and polychromatic cells, normally present in small numbers in the peripheral blood of rabbits, were increased. Platelets were difficult to find in blood smears at about the 9th to 11th days.

Rabbits given irradiation followed by bone marrow

Many of the marrow-treated animals behaved in a similar fashion to those not receiving marrow, but 2 animals in the group given 800 r, 5 animals in the group given 900 r and 10 animals in the group given 1000 r, showed a rapid rise in the total leucocyte count beginning at 3–5 days and reaching about 6000 cells per cu. mm. at 9 days (Fig. 9, 10 and 11). At the same time significant numbers of heterophils bearing characteristic nuclear " drum sticks " appeared in the peripheral blood indicating persistence and function of the transplanted marrow. These steadily increased until about the 14th day after which they remained fairly constant at an average of 1 per 18 heterophils. The total leucocyte count remained a little above normal until death or the end of the experiment.

Lymphocyte regeneration in these cases, while slower and less dramatic than heterophil recovery, was still appreciably more rapid, and a far less severe anaemia developed, than in those animals where the marrow transplant was unsuccessful or in the controls. Nucleated erythrocytes and polychromatic cells were not prominent and platelets were always to be seen.

The 2 animals in the 800 r group, 1 out of 5 in the 900 r group, and all 10 animals in the 1000 r group, despite their successful marrow transplants and haemopoietic recovery, died (Table II). Heterophils showing female sex chromatin were continually present in the 4 surviving animals with marrow transplants until the termination of the experiment at 60 days.

TABLE II.—*Time of Death of Animals Receiving a Successful Marrow Transplant after Total Body X-irradiation*

X-ray dosage	Time after irradiation (days)															
	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
800 r .	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—
900 r .	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
1000 r .	—	2	—	2	2	1	—	—	—	—	1	—	—	1	1	—

Pathology

Deaths occurred at two main periods, the first immediately after irradiation, and the second 7–15 days later.

Following X-irradiation, all animals showed shock-like prostration with loss of abdominal tone, excess moisture about the eyes and muzzle and diarrhoea. Of the animals exposed to 900 r or 1000 r 15–20 per cent died in this condition. There was no evidence that marrow treatment affected this immediate mortality

in any way. At autopsy there was little to be seen beyond pulmonary congestion and petechial haemorrhages in thymus and other lymphoid tissue.

Survivors recovered in 3–8 hr., but they ate and drank very little in the following days and lost weight rapidly. About 7 days after irradiation they became lethargic, lost fur, had diarrhoea, sometimes bled from the nose and mouth and many died in the next few days. In this group distension of the stomach with food was common at autopsy, often accompanied by gastric ulceration and perforation (30 per cent of those dying after 900 and 1000 r). Haemorrhage into the gastro-intestinal tract was also a common finding (25 per cent of those dying after 900 and 1000 r). Haemorrhage, however, was not found in those with successful marrow transplants.

In all animals the thymus shrank to such an extent in a few days that it was difficult to find at autopsy and in those dying early the spleen also was atrophic. This reduction in size was significant in spite of the great range in size of untreated spleens and considerable variation among those irradiated.

Microscopy

Rabbits exposed to irradiation without marrow treatment

Bone marrow.—In those dying there was destruction of the majority of the haemopoietic cells of the femoral bone marrow and their replacement by a gelatinous and fatty type of marrow (Fig. 2). The degenerative effects increased fairly steadily until about the 5th to the 7th day, when an abortive attempt at myelopoiesis was indicated by the appearance of a few tiny groups of young myelocytes. These had largely disappeared in those animals dying at 9 days or later, when a similar abortive attempt at erythropoiesis was sometimes present. Those animals that survived showed essentially normal marrow at 60 days.

Spleen.—The white pulp was atrophic with destruction of the germinal centres bringing the reticular cells into prominence (Fig. 5). The first indication of regeneration of lymphocytes was seen in animals dying at 9 days, and regeneration was well established at 14 days, and was complete in those killed at 60 days. Haemosiderin was prominent in the red pulp in those dying between 7 and 14 days.

Lymphoid tissue.—The lymphocytes of the thymic cortex were destroyed, the lobules shrunken and the stroma contracted to form a solid sheet of cells in the region where the cortex had been. Regeneration was slow but restoration was complete in those animals killed at 60 days. In the mesenteric lymph nodes there was massive destruction of lymphocytes with replacement of the lymphatic nodules by densely packed " epithelioid " cells. In animals surviving beyond 8 days these groups of cells became gradually smaller. Regeneration was slow and the germinal centres were not all restored to normal in those animals killed at the end of the experiment.

Rabbits given total body irradiation followed by bone marrow

Bone marrow.—In those animals where female heterophils appeared in the peripheral circulation there was early recovery of the marrow. Intensely active myelopoiesis as shown by masses of early and late forms with frequent mitotic figures, and erythropoiesis with large numbers of erythroblasts in all stages were seen in 2 animals dying at 9 days after 1000 r. Many young megakaryocytes were also present (Fig. 3). This picture was in striking contrast to the atrophic

marrow present in those control irradiated animals dying at this period. Animals surviving longer showed a hyperplastic marrow. In some plasma cells were prominent. The marrow of those killed at 60 days appeared normal. Careful search in bone marrow smears revealed the very occasional heterophil with a nuclear " drum stick " (Fig. 7).

Spleen.—Regeneration with restoration of lymphoid nodules and germinal centres occurred earlier and was complete sooner than in the corresponding controls (Fig. 6). Haemosiderin was again present in the red pulp but less obvious than in the non-marrow-treated animals. Ectopic haemopoiesis was either absent or slight.

Lymphoid tissue.—The initial phase of destruction and phagocytosis in the mesenteric lymph nodes was followed by rapid regeneration, plasma cells being found in the medullary cords together with masses of medium-sized lymphocytes. Germinal centres were restored in animals surviving longer than 11 days. The thymus, although regenerating more rapidly than in the controls, was slow in returning to normal.

The foregoing description of changes in bone marrow, spleen and lymphoid tissue is confined to those animals in which there was a successful marrow transplant. In animals where the transplant failed, regeneration was essentially no different from that observed in animals from corresponding groups which had not received marrow intravenously, and in some there was evidence of a terminal massive bacteraemia.

DISCUSSION

In this investigation intravenous homologous bone marrow given immediately after exposure of rabbits to 900 r total body X-irradiation is shown to be capable of enhancing their survival, whereas similar marrow treatment elicited only slight prolongation of life at 1000 r and had no appreciable effect at 800 r (LD_{50}). With mouse bone marrow, however, Lorenz, Congdon and Uphoff (1952) were able to reduce the mortality of X-irradiated mice (900 r) from 100 per cent in the controls to 20 per cent in mice injected with bone marrow from other individuals of the same inbred strain. Mouse marrow therefore seems to exert greater protective activity than marrow from the rabbit ; it also affords protection against radiation levels at which rabbit bone marrow is apparently ineffective. There are probably two reasons for this disparity in the response of the rabbit

EXPLANATION OF PLATES

FIG. 1.—Femoral bone marrow from normal male rabbit. Haematoxylin and eosin. × 250.

FIG. 2.—Aplastic marrow 9 days after 1000 r total body X-irradiation. The venous sinuses are greatly dilated and contain red cells. Haematoxylin and eosin. × 250.

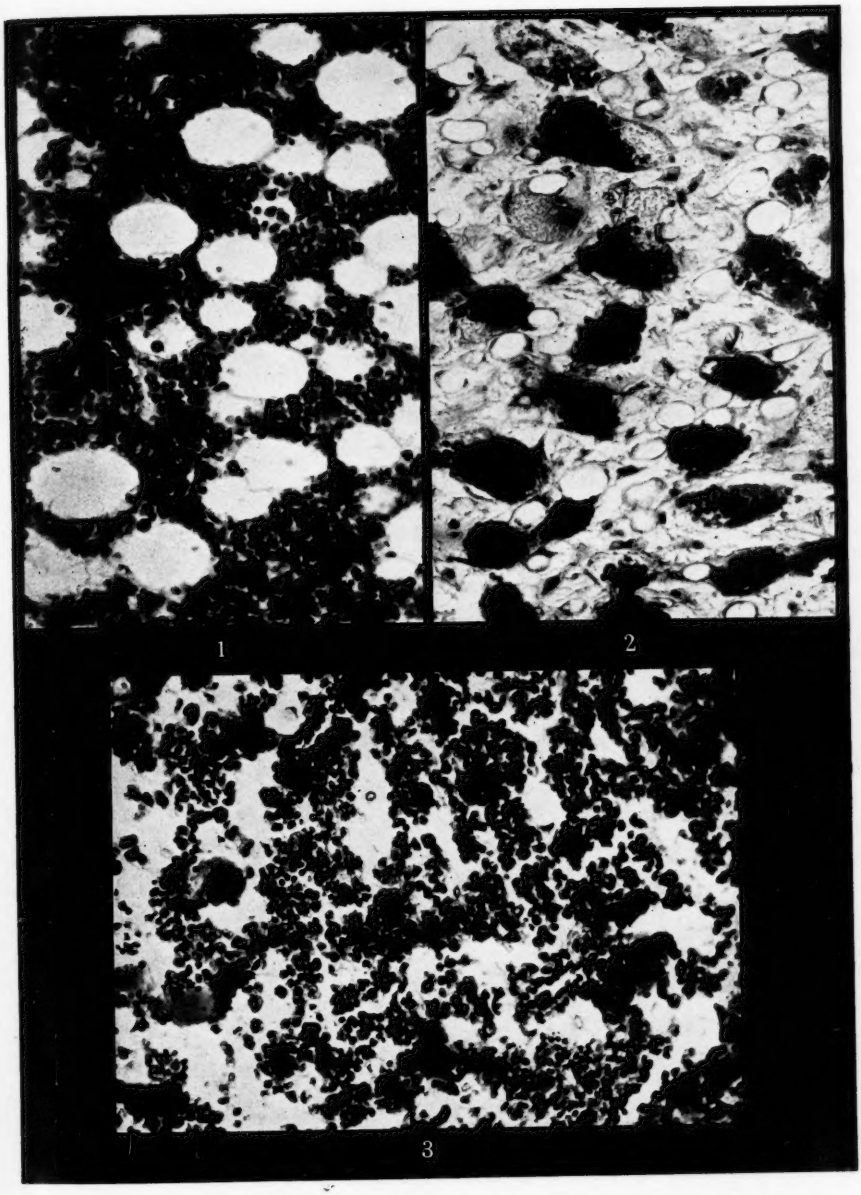
FIG. 3.—Repopulation of male marrow spaces by transplanted female marrow cells 9 days after 1000 R total body X-irradiation. Haematoxylin and eosin. × 250.

FIG. 4.—Spleen from normal male rabbit. Haematoxylin and eosin. × 200.

FIG. 5.—Atrophic spleen 9 days after 1000 r total body X-irradiation. Lymphoid nodules are absent while reticular cells and stroma are prominent. Haematoxylin and eosin. × 200.

FIG. 6.—Spleen from a rabbit 9 days after 1000 r X-irradiation and marrow treatment. There is early regeneration of the lymphoid nodules. Haematoxylin and eosin. × 200.

FIG. 7.—Heterophil showing female nuclear " drum stick " in marrow of male rabbit 21 days after 1000 r total body X-irradiation and marrow treatment. Wright's stain. × 800.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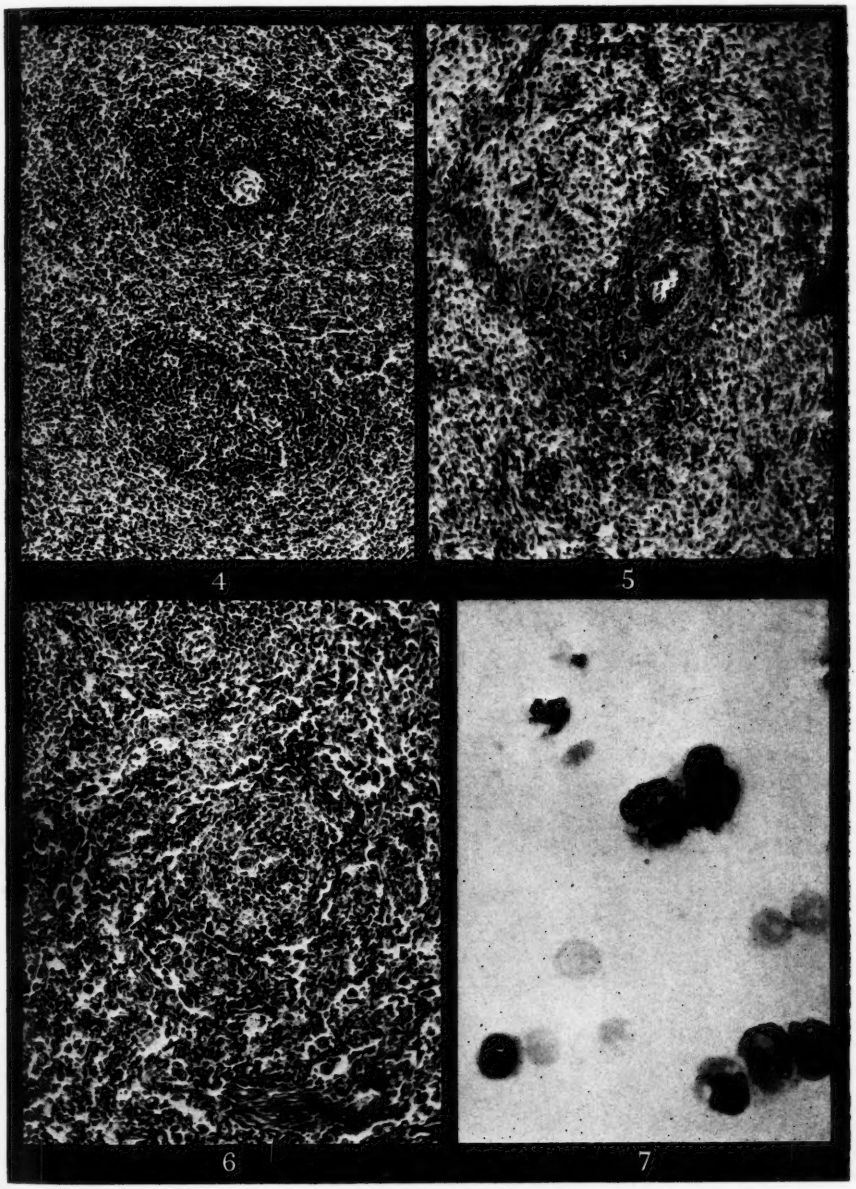

Porter.

and the mouse to bone marrow treatment following total body X-irradiation at about the lethal dose level :

1. A species difference in the relative radiosensitivity of the gastro-intestinal tract.

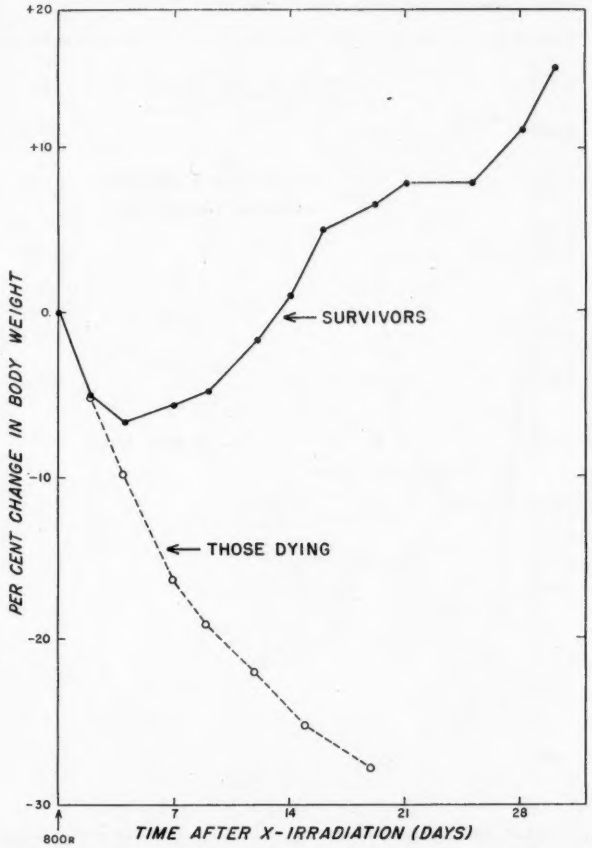

FIG. 8.—Weight curve of surviving animals compared with that of those dying following 800 r total body X-irradiation.

The exceptional sensitivity of the gastro-intestinal tract to ionizing radiations, and variations in species susceptibility, have long been recognized (Regaud, Nogier and Lacassagne, 1912 ; Warren and Whipple, 1923). In the present experiment, shock, early deaths, marked diarrhoea, gastric distension, ulceration and perforation were prominent following lethal doses of X-rays (900 r and 1000 r). The high incidence of discrete gastric ulceration with perforation in the rabbit after large doses of irradiation has been noted previously (Engelstad, 1938). Protection against all these complications can be achieved by lead

shielding the abdomen (Porter, unpublished observations), while lead protection of the exteriorized small intestine alone will prevent the initial shock, diarrhoea and early deaths (Billings, Dowdy, Burlingame and Lamport, 1951). However, the animals will still die later largely from haemorrhage and infection due to failure of adequate and sufficiently rapid haemopoietic recovery.

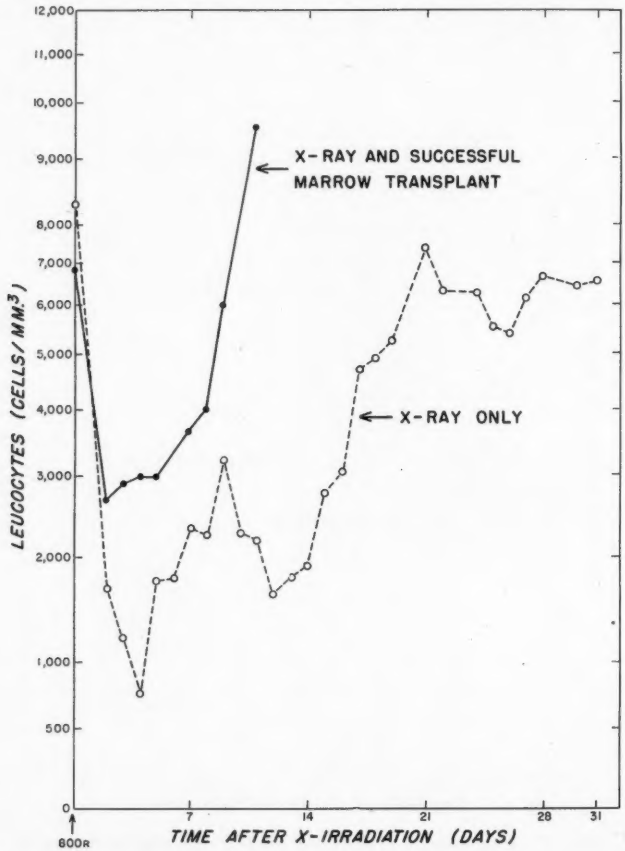

FIG. 9.—Effect of successful homologous marrow transplant on recovery of leucocytes in peripheral blood of rabbits after 800 r total body X-irradiation.

The dose necessary to produce comparable gastro-intestinal complications in the mouse is much higher, manifestations of the intestinal complex only appearing in the supralethal range (Bond, Silverman and Cronkite, 1954). It appears, therefore, that at any given radiation dose in the lethal region, the rabbit shows a larger component of gastro-intestinal injury than the mouse and that these lesions may lead to the death of some animals despite a successful marrow transplant and haemopoietic recovery.

A similar species difference in the radiosensitivity of the gut probably accounts for the relative ineffectiveness of marrow injections in reducing radiation mortality in rats as compared with mice after exposure to lethal doses (Fishler *et al.*, 1954). These observations emphasize the limitations of post-irradiation bone marrow

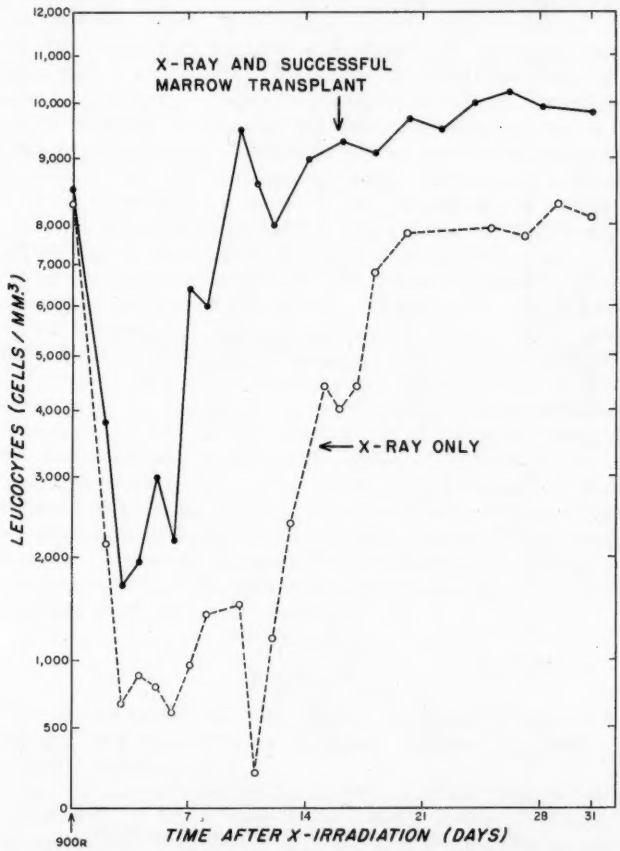

FIG. 10.—Effect of successful homologous marrow transplant on recovery of leucocytes in peripheral blood of rabbits after 900 r total body X-irradiation.

treatment in the rat and the rabbit and focus attention on systems other than the blood-forming tissues which are also basic to recovery from radiation injury.

2. The closeness of the genetic relationship between host and marrow donor.

It has become obvious from several studies that the length of post-irradiation survival provided by bone marrow, and the ease with which reproducible results can be obtained, are proportional to the genetic relationship of the marrow donor and the irradiated recipient. The best results are obtained when the bone marrow

donor belongs to the same inbred strain as the irradiated animal, as in Lorenz's experiment on mice quoted above. Therefore it should be emphasized that the rabbits used in these experiments were not inbred in the genetic sense and that the marrow was homologous, not isologous.

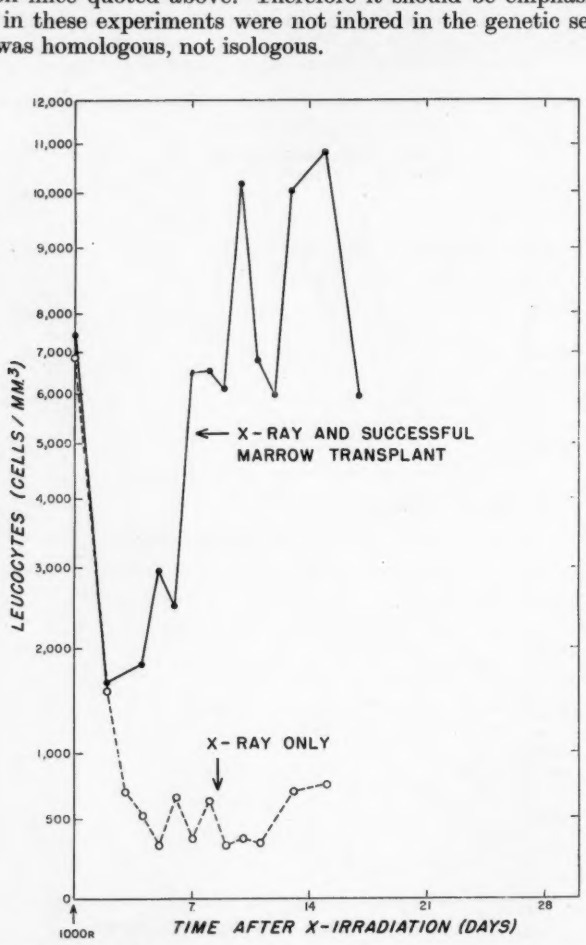

FIG. 11.—Effect of successful homologous marrow transplant on recovery of leucocytes in peripheral blood of rabbits after 1000 r total body X-irradiation.

The data obtained in these experiments add to the considerable evidence recently accumulated (Ford *et al.*, 1956; Mitchison, 1956; Makinodan, 1956; Nowell, Cole, Habermeyer and Roan, 1956; Merwin and Congdon, 1956; Lindsley, Odell and Tausche, 1955) that in irradiated animals treated with homologous or heterologous marrow, the introduced cells survive and repopulate the host's depleted haemopoietic tissues. The " marker " technique used here has proved a simple method with one main disadvantage that on the average only 1 in 18

heterophils are found with typical " drum sticks "; and in the early recovery phase at about 3–4 days post-irradiation, when heterophils are not only infrequent but many are still immature, this makes searching for labelled cells a tedious task. However, examination of smears from the haematocrit buffy coat is a considerable help at this time. A great advantage of the method is that it permits detection of successful homologous marrow transplants without any necessity for killing the animals. The immunogenetic erythrocyte marker technique of Lindsley *et al.* (1955) also has the same advantage, but in many other ways it is less simple and depends on a supply of animals of specific antigenic type. As it is probable that a similar morphological sex difference will be shown to be present in the neutrophils of many mammalian species other than the rabbit, the method described in this paper may prove useful in similar work with larger animals.

It will be noticed that with increasing dosage of irradiation above the LD_{50} (800 r) there was a progressive increase in the proportion of successful marrow transplants. This is presumably related to the decreasing ability of the host to mount an immunological response to foreign tissue antigens.

Previous work with rabbits has tended to emphasize factors in post-irradiation protection other than haemopoietic cellular repopulation. Jacobson, Marks and Gaston (1956) have found that baby mouse liver or spleen cells increase survival of rabbits exposed to 900 R without producing any effect on haematological recovery. Hilfinger and Ferguson after a preliminary paper (1951) reporting the more rapid return of leucocytes in the peripheral blood of marrow-treated irradiated rabbits, have more recently with Riemenschneider (1953) stated that haemopoietic recovery is not hastened and that equally beneficial effects on survival can be obtained with a non-cellular fraction of the marrow suspension. Finally, Allen, Wardell and Clay (1956) have found enhancement of survival after 1000 R by intraperitoneal injections of fresh cell-free splenic plasma. These reports suggest that there might be some factor in addition to cellular repopulation operative in post-irradiation protection of the rabbit by spleen and marrow cell suspensions. At present this possibility cannot be excluded.

SUMMARY

Male rabbits were exposed to 800 r (LD_{50} 30 days), 900 r and 1000 r total body X-irradiation and subsequently injected intravenously with homologous bone marrow from a female donor.

In 15 per cent of the animals receiving 800 r, 38 per cent of those receiving 900 r and 62 per cent of those exposed to 1000 r, heterophils bearing nuclear chromatin " drum sticks " characteristic of the female appeared in the peripheral circulation within 3–4 days. These cells rapidly increased in number restoring the the total leucocyte count to normal or above normal levels, indicating persistence and function of the marrow implants. At 60 days female heterophils were still present in 4 male animals. Microscopical studies confirmed the bone marrow repopulation and showed more rapid recovery of lymphoid tissue in the treated animals.

Marrow treatment reduced the mortality of the rabbits exposed to 900 r from 92 per cent to 54 per cent, but it was ineffective after 800 r and only slightly prolonged the mean survival time of the animals dying after 1000 r. The 100 per cent mortality following 1000 r despite successful marrow transplantation and

haemopoietic recovery, seemed to be largely due to the relative radiosensitivity of the gastro-intestinal tract.

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THE INFLUENCE OF CORTICOTROPHIN AND CERTAIN CORTICOSTEROIDS ON POPULATIONS OF *MYCOBACTERIUM TUBERCULOSIS* IN TISSUES OF MICE

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THE modification of host-parasite relationships by corticotrophin and corticosteroids has been studied extensively in many mammalian species including man with the general conclusion that there is an enhancement of the infective process to the detriment of the host. Most aspects of this field are not clearly understood, however, and although most observations have indicated that the effects of the hormones are deleterious, this may not necessarily always be the case. For example, Lurie, Zappasodi, Dannenberg and Swartz (1951) have demonstrated that experimentally infected rabbits of a highly susceptible strain when treated with cortisone react with certain of the tissue responses characteristic of natively resistant rabbits. At the same time, very large numbers of acid-fast bacilli were observed in the pulmonary lesions of the animals receiving the hormone. In terms of the total well-being of the host these two phenomena represent forces which are acting in opposite directions and the net effect would depend on which predominated. It has also been noted that there is much species variation in action of the hormones both in tuberculosis and in other fields of study. The effects of the different hormones are not uniform. For example, though there is general agreement that increased microbial growth *in vivo* occurs during administration of cortisone, hydrocortisone and their derivatives, there is no unanimity concerning the effect of corticotrophin. In many animals such as the rat and the mouse, the latter produces definite pharmacological effects (Speirs and Meyer, 1949), but causes little or no enhancement of infection, as was shown for mice by Kass, Ingbar, Lungdren and Finland (1951) and for hamsters by Shwartzman (1950).

Knowledge of the action of the hormones in experimental tuberculosis has been obtained mainly from the study of their effect on the survival of infected animals and from the changes in tissue architecture. Much valuable information has been gained with these methods but they have two important disadvantages. The survival time of hormone-treated animals may be diminished by non-tuberculous infections ; and measurement of change in size of the population of tubercle bacilli by microscopy has been made only by approximate estimates and is necessarily inexact. It seemed desirable, therefore, to study this question by the use of more precise techniques for observing the dynamics of the host-parasite relationship. Tubercle bacilli in mouse tissues can be counted with a high degree

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of reproducibility by the method devised by Fenner, Martin and Pierce (1949). This method has been used extensively to measure changes in host resistance (Pierce, Dubos and Schaefer, 1953) and the influence of antimicrobial drugs in experimental infections both with tubercle bacilli (McCune and Tompsett, 1956; McCune, Tompsett and McDermott, 1956) and with staphylococci (McCune, Dineen and Batten, 1956). Its use for the study of the effect of corticotrophin and corticosteroids on tuberculosis in mice is reported here. In an accompanying report (Batten and McCune, 1957) observations are presented on the results obtained when antituberculous drugs were administered in various combinations with cortisone or corticotrophin.

MATERIALS AND METHODS

The methods used in this study are similar to those previously reported from this laboratory (McCune and Tompsett, 1956) and will be described only briefly.

Male albino mice (Webster Swiss strain) weighing 18–20 g. were obtained 1 week before infection and were housed in metal cages—not more than 10 animals to a cage. They were fed on standard mouse pellets and water intake was unrestricted.

The mice were inoculated intravenously with 0·2 ml. of a 1/10 dilution of a 7-day Tween-albumin culture of *Mycobacterium tuberculosis hominis* (H37Rv) in 0·1 per cent bovine albumin in saline. In each experiment the size of the inoculum was estimated by surface viable counts; there were approximately $1–3 \times 10^6$ cultivable units per ml. in the inocula used for infecting the animals. The resulting infection was a chronic generalized tuberculosis, predominantly pulmonary, which proved fatal from 5–7 months after infection.

The preparation of hormones and the vehicles used were as follows:

Corticotrophin: (Acthar-Armour) in saline and in hydrolysed gelatin vehicle; hydrocortisone acetate (Merck) saline suspension; cortisone acetate (Schering) aqueous crystalline suspension; hydrolysed gelatine (Armour). All of the preparations were given by intramuscular injection into the thigh, starting on the day of infection and continuing until the end of the experiment.

Twenty-four hours after inoculation and at intervals up to 8 weeks, 3–5 animals in each treatment group were killed with chloroform and autopsies were performed aseptically. The lungs and spleen were removed and their volumes measured by displacement of 5 ml. of diluent (2 per cent bovine albumin in saline) in graduated centrifuge tubes. The tissues were then transferred to Pyrex tubes and emulsified with a plastic (Teflon) grinder. Three 0·02 ml. aliquots of appropriate dilutions of the homogenates were cultivated on oleic acid albumin agar. Colony counts were carried out by two independent observers after 2 and 4 weeks of incubation at 37°. Taking into account the various dilution factors the number of "cultivable units" of tubercle bacilli was estimated for each unit (ml.) of tissue. The results were expressed logarithmically and are presented graphically.

With this method it is theoretically possible to detect 70–90 tubercle bacilli present in either lung or spleen. For detection of fewer organisms, the technique may be modified by alteration of dilution factors so that it is theoretically possible to detect 1–3 cultivable units in either of the two organs. As this modification becomes relevant only in the succeeding paper it will be discussed there.

RESULTS

A preliminary experiment was designed to see if corticotrophin exerted an enhancing effect on the microbial populations. Attempts were also made to discover the most satisfactory dose and preparation of the hormone and the most effective frequency of administration.

Three mice were killed 24 hr. after infection for determination of initial population levels. Five mice were killed 20 days later in each of the 10 groups which are specified in the Table. It will be noted that two dosage levels (4·0 and 0·4 mg./day) were used and were administered in two vehicles: 0·2 ml. saline

TABLE.—*The Effect of Altering Time-dose Relationships and the Method of Corticotrophin Administration on Populations of* Myco. tuberculosis (*H37Rv*) *in Lungs and Spleens of Mice. Infecting Inoculum :* 1·8 × 10^6 *Cultivable Units Tubercle Bacilli*

Daily regimen				Viable units tubercle bacilli (H37Rv)* per ml. tissue. × 10^6	
Dose (mg.)	Number of injections	Vehicles		Lung	Spleen
—	—	—	1 day	0·3	1·9
—	—	—	21 days	36	7
—	2	S		36	13
—	2	G		33	17
0·4	2	S		36	8
0·4	4	S		500	31
0·4	1	G		40	12
0·4	2	G		400	45
4·0	2	S		66	18
4·0	4	S		700	200
4·0	1	G		300	53
4·0	2	G		1500	300

* Average of 5 animals in each group.
† S = saline ; G = hydrolysed gelatin.

given twice or four times daily and 0·2 ml. hydrolysed gelatin given once or twice daily.

Animals in two groups received the same volume of one or the other vehicle alone and were included together with infected untreated animals as controls.

The results are presented in the Table. There was a 20-fold increase in the populations of tubercle bacilli in both the lung and the spleen of the animals which received 4·0 mg./day of corticotrophin when 4 doses were given daily in saline or twice daily in gelatin. In contrast, no significant effects on the microbial populations were produced by the 0·4 mg. dose of hormone given once daily in gelatin or twice daily in saline, nor by the vehicles alone. The increase in populations in the groups on intermediate dosage did not achieve significance on statistical analysis because of the small numbers of animals used. Nevertheless, there was a definite trend throughout the groups which indicated that an increase in population size resulting from corticotrophin administration depended not only on total daily dose, but also on frequency of administration. No animals died in the course of this experiment.

In view of these results an effort was made in all subsequent experiments to maintain a continuously high concentration of hormone in the blood. For this purpose a dose of 2 mg. of corticotrophin in gelatin administered twice daily was chosen.

Observations on Microbial Populations Influenced by Cortisone and Corticotrophin over an Eight-week Period

In the next experiments in which the effects of cortisone and corticotrophin on the populations of tubercle bacilli were compared, the same method was extended over an 8-week period. Data were gathered from 4 identical experiments and the results are presented in Fig. 1 and 2. Each symbol represents the

mean of the logarithm values of the populations in the lungs and spleens of 9–18 animals killed at the intervals of time indicated. The dose of corticotrophin was 2·0 mg. administered in gelatin vehicle twice daily by the intramuscular route;

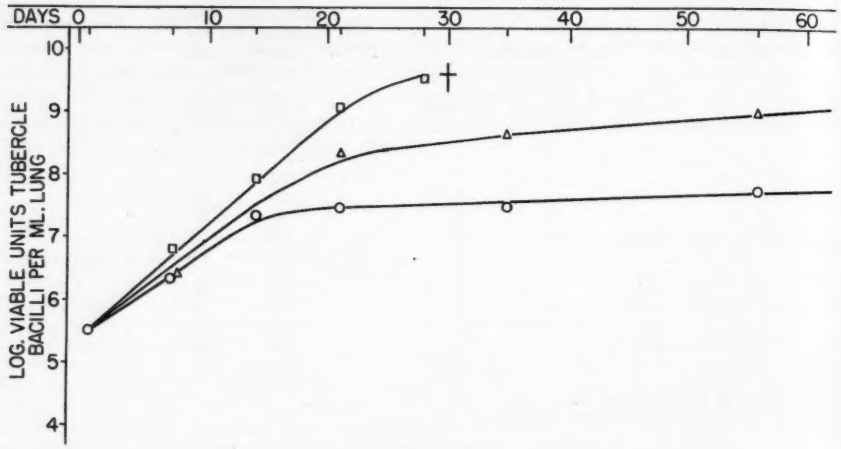

FIG. 1.—Influence of corticotrophin and cortisone on populations of *Myco. tuberculosis* (H37Rv) in mouse lungs. Infecting inoculum: $3{\cdot}4 \times 10^6$ cultivable units tubercle bacilli.

○——○ control.
△——△ cortisone.
□——□ corticotrophin.

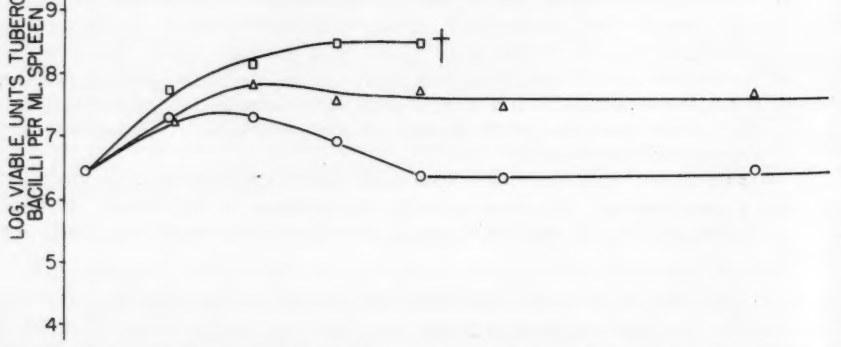

FIG. 2.—Influence of corticotrophin and cortisone on populations of *Myco. tuberculosis* (H37Rv) in spleens of the same animals whose lung populations are shown in Fig. 1.

○——○ control.
△——△ cortisone.
□——□ corticotrophin.

0·1 mg. cortisone acetate was given as a saline suspension once daily by the same route.

Infected untreated mice

The changes which occurred in the size of the populations of tubercle bacilli on both lungs and spleen throughout the experimental period were of the same nature as those previously described from this laboratory (McCune and Tompsett, 1956).

In the spleen, there was an initial increase of the populations of tubercle bacilli with a fall to the original census within a period of 3 weeks after institution of infection. The census stabilized at this point (approximately 10^6 tubercle bacilli per ml. of spleen) and remained constant throughout the remainder of the experiment. In the lung there was a steady increase in the population to about 10^8 organisms per ml. 8 weeks after infection. No animals died in this group.

Infected mice which received corticotrophin

In both the spleen and lung of the mice which received corticotrophin there was a considerable rise in the populations of tubercle bacilli when compared with those of the untreated animals (Fig. 1 and 2). In the spleen there were approximately 5×10^8 organisms after 3 weeks. Despite this great increase in population the numbers stabilized as in control animals, although at a high level. In the lungs of the same animals there was a rapid rise to a very high level (5×10^9) after the same period of time. As may be seen in Fig. 3 no animals survived beyond the end of the fourth week of infection.

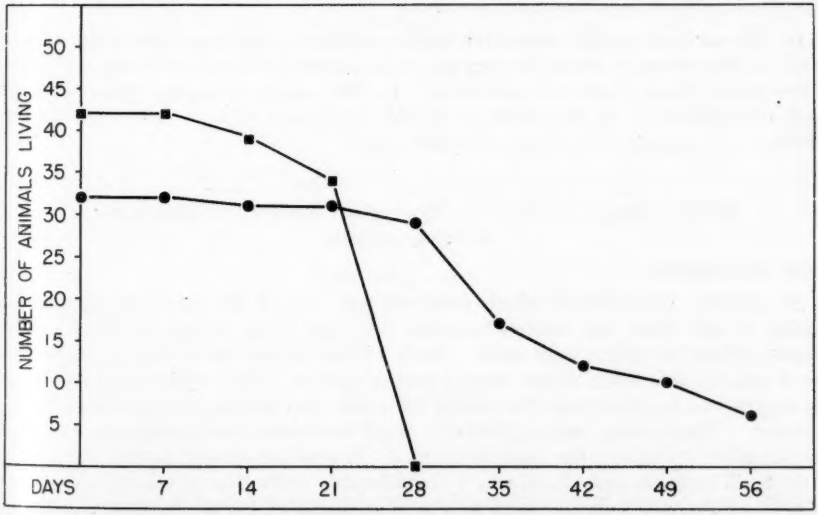

FIG. 3.—Influence of corticotrophin and cortisone on death rate of mice infected with *Myco. tuberculosis* (H37Rv).

■——■ animals given corticotrophin.
●——● „ „ cortisone.

Infected mice which received cortisone

The behaviour of the populations of tubercle bacilli in the lungs and spleens of the group of mice which received cortisone is also presented in Fig. 1 and 2. In both organs, there was a greater than 10-fold increase in population size above that which occurred in the control animals. In the spleen there was no fall in population after an initial increase to about 5×10^7 organisms. At this level stabilization occurred, and persisted throughout the latter part of the experiment. In the lung there was a marked initial multiplication which diminished in degree as the experiment progressed. The populations of tubercle bacilli reached a level of about 10^9 organisms at the end of 8 weeks.

There was a gradual increase in death rate beginning at the end of the fourth week. Seventy per cent of the animals in this group which received cortisone died within the 8-week period (Fig. 3).

Comparison of Cortisone and Hydrocortisone

The comparative effects of cortisone and hydrocortisone on the populations of tubercle bacilli are presented in Fig. 4 and 5. Both hormones were administered intramuscularly in a dose of 0·1 mg. daily. Each symbol in the Figures represents the population of tubercle bacilli in the lung or spleen of a single animal.

The population trends in the animals which received cortisone and the animals which received no treatment are similar to those observed in the 8-week experiment previously described. The present experiment, however, was continued for 12 weeks. No cortisone-treated animals lived longer than 8 weeks.

Infected mice which received hydrocortisone

In the animals which received hydrocortisone, the populations of tubercle bacilli in the spleen showed an increase which closely followed the trend observed in the mice which received cortisone. In the lung, however, there was very rapid multiplication of the tubercle bacilli to a level of 5×10^9 4 weeks after infection, no animals surviving after this time.

Tissue Changes in Infected Mice which Received Corticosteroids or Corticotrophin

Gross examination

At autopsy all animals which received any one of the three hormones were smaller in size than the control animals. In the lungs widely scattered lesions greyish-yellow in colour were seen. Some of the lesions were discrete, measuring 2 or 3 mm. in diameter, others were lobar in extent. The intervening lung tissue was bright red in colour and the lungs were firm and tended to sink on immersion in water. The spleens were extremely small and were one-quarter or less of the volume of the spleens in the control animals. It was also noted that the thymus was small in all animals and there was a considerable reduction of retroperitoneal fat. All of these changes were most marked in the animals which received corticotrophin. The adrenal glands in these latter were hypertrophied and presented a noticeable contrast to the atrophied spleens. In the animals which received hydrocortisone or cortisone, the adrenal glands appeared normal or reduced in size.

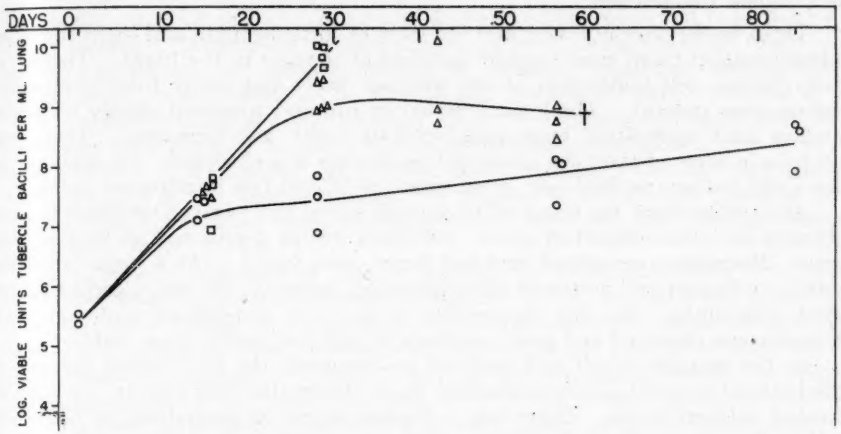

FIG. 4.—Influence of hydrocortisone and cortisone on populations of tubercle bacilli (H37Rv) in mouse lungs. Infecting inoculum: $3 \cdot 2 \times 10^6$ cultivable units tubercle bacilli. No animals survived after treatment for 28 days with hydrocortisone or 56 days with cortisone.

○——○ control.
□——□ hydrocortisone.
△——△ cortisone.

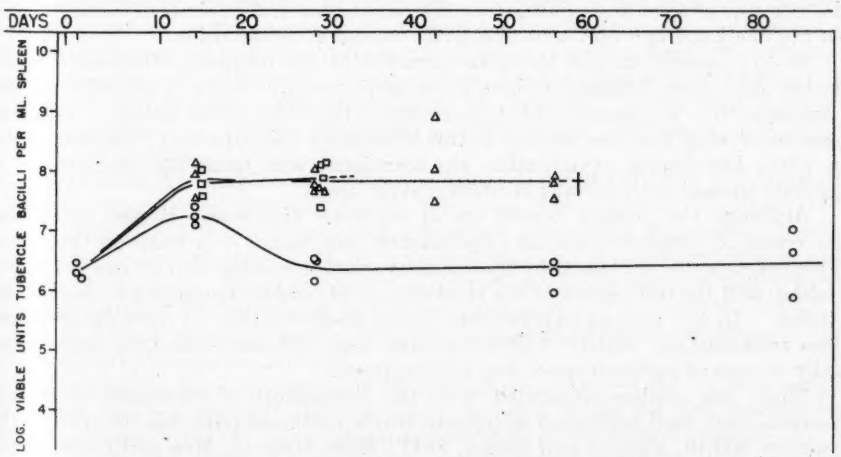

FIG. 5.—Influence of hydrocortisone and cortisone on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung populations are shown in Fig. 4.

○——○ control.
□——□ hydrocortisone.
△——△ cortisone.

Microscopic examination

Three weeks after infection and the start of corticotrophin and hydrocortisone administration there were marked histological changes in the lungs. There was mononuclear cell infiltration of the alveolar walls and many focal pneumonic lesions were present. The lesions varied in size and consisted chiefly of mononuclear and epithelioid type cells without giant cell formation. There was necrosis in some of the focal areas, but no fibrosis was observed. On staining by the Ziehl-Neelsen method very great numbers of acid-fast bacilli were visible.

At a similar time, the lungs of the animals which had received cortisone showed changes like those described above, but much less in degree and no necrosis was seen. Moderate numbers of acid-fast forms were found. At 8 weeks after the start of infection and cortisone administration, however, the histological changes were pronounced and the pneumonic areas were widespread and confluent. Necrosis was observed and great numbers of acid-fast bacilli were visible.

In the animals which had received no hormone, the histological changes in the lungs at 3 weeks closely resembled those observed at this time in the animals treated with cortisone. There was a greater degree of generalized mononuclear cell infiltration in the infected control group but only scanty acid-fast bacilli were seen.

DISCUSSION

The results of these experiments permit the conclusion that in albino mice infected with the virulent human strain of *Myco. tuberculosis* (H37Rv), corticotrophin, cortisone and hydrocortisone in appropriate doses gave rise to a significant increase in the microbial populations in lungs and spleens. Furthermore, there did not appear to be any qualitative difference between the effects of corticotrophin on the one hand and cortisone and hydrocortisone on the other.

In the present studies the determination of the numbers of tubercle bacilli within the organs appeared to be a much more sensitive index of the effects of the hormones than the studies of the character of the tuberculous lesions. This is in agreement with previous studies in this laboratory (McCune and Tompsett, 1956) in which histological examination was correlated with microbial enumeration in animals treated with various antituberculous drugs.

Although the present results are at variance with many studies concerning the effect of corticotrophin on experimental infections, it is believed that these differences can be explained by the species variation in adrenal response to corticotrophin and the differences of the time-dose relationships employed in the various studies. In the present experiments it was observed that by varying the time-dose relationships, widely different results were obtained when the same total daily dosage of corticotrophin was administered.

There are studies concerned with the depression of resistance to several bacterial and viral infections in mice in which corticotrophin was observed to be inactive (Smith, Murphy and Mirick, 1951 ; Kass, Hechter, Mou and Lurie, 1955). In one experiment (Southam and Babcock, 1951), however, corticotrophin was given in a dose of 4 mg. in gelatin vehicle at 12-hour intervals and was considered to depress resistance to the West Nile virus.

In a review (Johnson and Davey, 1954) of the influence of steroids on tuberculosis in other animal species only one instance could be found where cortico-

trophin had enhanced the experimental infection : namely in ocular tuberculosis in rabbits. In this instance Bunn and Drobeck (1952) observed that there was more extensive progression of the disease, greater destruction of tissue, and greater frequency of distant spread in animals receiving corticotrophin. Contrariwise, in two studies (Le Maistre and Tompsett, 1951 ; Weimer *et al.*, 1953) in which relatively high dosages of corticotrophin were used and when injections were given at 6-hour intervals in guinea-pigs, no effects were observed on the extent of the lesions. In rabbits, however, using relatively high doses of corticotrophin in the same frequency, the progress of the disease appeared to be no more rapid than when cortisone was given in 1/10 of the dose (Wanzer, Morgan and Smith, 1954).

With specific reference to murine tuberculosis there have been few studies concerned with the influence of corticotrophin. In one report it was stated that when a dose of 0·5 mg. of corticotrophin was administered in saline, the survival of mice infected with *Myco. tuberculosis* (H37Rv) did not vary from that observed in infected but untreated animals (Youmans and Youmans, 1954). In another experiment (Swedberg, Dahlstrom and Luft, 1951) it was considered that the survival time of mice infected with a virulent strain of *Myco. tuberculosis* was reduced significantly. In this latter study, however, mice receiving control injections alone were not used. In a preliminary study (Hobby, Auerbach and Lenert, 1955) in white mice infected intracerebrally with the virulent Vallee strain of *Myco. tuberculosis* and given 1·2 units of corticotrophin in 5 per cent beeswax thrice weekly, there was a reduction in survival time from 43 to 23 days and the inflammatory process in the meninges progressed to a state of necrosis. Necrosis was also observed in animals receiving cortisone and hydrocortisone, but not in infected animals not receiving hormones.

As previously suggested the apparent differences noted here may therefore reflect a species variation in adrenal response to corticotrophin. In some of the experiments quoted, however, it is clear that little or no effect was observed because of inadequate and ephemeral concentrations of the hormone in the blood. The present findings confirm this and support the idea that adrenal stimulation by corticotrophin is a purely quantitative phenomenon, varying in degree from species to species.

That there is a qualitative variation in adrenal output naturally and in response to corticotrophin in different species has been demonstrated by Bush (1953). Kass *et al.* (1955) have shown that variations may occur in adrenal output in response to infection within the same species according to the susceptibility of the strain of the host. The significance of output of various steroids under varying conditions from the adrenal is as yet unknown nor is it yet certain that an undefined steroid is not the active principle in the enhancement of infection which is mediated through the adrenal.

Although no data are available concerning the nature of the adrenal secretion in mice, the predominant adrenal steroid secreted in the rat and the rabbits is corticosterone. It has been demonstrated that either hydrocortisone or corticosterone has been the predominating steroid released in all the animals thus far studied. Corticosterone appears to be relatively ineffective as an anti-inflammatory agent (Long and Spensley, 1954). Cortisone and hydrocortisone exert effects in mice qualitatively similar to those of corticotrophin. It is thus suggested that hydrocortisone might be produced by the murine adrenal gland.

The results obtained in animals receiving cortisone in the present study agree with those reported previously (Hart and Rees, 1950; Solotorovsky, Gregory and Stoerk, 1951). In one report (Engbaek, Friis and Teilum, 1952) a 10-fold increase was observed in the populations of *Myco. tuberculosis* in the lungs of mice receiving cortisone; no rise occurred in the spleens of the same animals. In most species the same phenomenon is reported, a notable exception being the guinea-pig. In this animal, several workers (Le Maistre and Tompsett, 1951; Weimer *et al.*, 1953) found that neither cortisone nor corticotrophin caused any adverse effects on the disease.

It is of interest that hydrocortisone in the same dose exerted a far more marked effect on populations than cortisone. This observation merits further study as this method might prove useful in assessing derivatives of this series of steroids for their ability to enhance infection as distinct from their many other properties.

The mechanism of the effects noted remains unclear. The hormones may have altered the internal environment in such a way that extraordinarily high populations of tubercle bacilli were attained without causing early death. Such an environmental change could take the form simply of protecting host tissue against microbial influence and hence passively encouraging unrestricted bacterial proliferation. From the present experiments it seems likely that the situation is more complicated and that some inhibitory mechanism of the host is also being affected by the hormone. Otherwise it is difficult to explain why the same stabilization of the census noted in the untreated animals also occurred, although at a higher level, in the animals maintained on hormone. This population stabilization occurred at various levels depending on the organ or hormone involved.

The absolute height of the census of tubercle bacilli in certain experimental groups was remarkable and shows that the population in the fatal untreated infection is considerably lower than the maximal attainable level. Presumably, as mentioned above, this phenomenon represents a protective effect of hormone on the host tissues. Similar observations have been reported for the effects of cortisone on an influenza B viral infection in the chick embryo or in tissue culture (Kilbourne and Horsfall, 1951; Kilbourne and Tateno, 1953). In this situation, apparently simpler because no known factors of host resistance to infection have been found, it was proposed that the hormone postponed the death of the cell and hence higher populations could be tolerated.

In view of the rapidity of the effect of the steroids in the present studies it is unlikely that interference with acquired resistance played a significant rôle in bringing about the increase of the populations of tubercle bacilli. A direct effect of the hormones on tubercle bacilli *in vivo* cannot be excluded, but *in vitro* experiments concerned with this problem have yielded negative results.

SUMMARY

A study has been made of the influence of corticotrophin, cortisone and hydrocortisone on microbial populations (H37Rv) in the lungs and spleens of albino mice.

When these hormones were given the populations greatly increased, the survival time was shortened and the lesions in the tissues were modified. It also

appeared that the adrenal response to corticotrophin in the mouse was a function of the dose of the hormone and the method of administration, and that the method used might prove of value in assessing new derivatives of the adrenocortical steroids for their enhancing action on infective processes.

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THE INFLUENCE OF CORTICOTROPHIN AND CORTISONE WITH ANTITUBERCULOUS DRUGS ON POPULATIONS OF *MYCOBACTERIUM TUBERCULOSIS* IN TISSUES OF MICE

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IN the preceding paper (Batten and McCune, 1957) it was reported that cortisone, hydrocortisone and corticotrophin gave rise to an enhancement of the tuberculous process in the lungs and spleens of albino mice. This conclusion was based on the greater multiplication of tubercle bacilli which occurred in the tissues of animals receiving these hormones than in the infected untreated mice. Observations on survival times and tissue changes provided additional evidence.

Whether cortisone or corticotrophin under such circumstances would bring about an increase or decrease in antituberculous drug effectiveness is not predictable on theoretical grounds. On the one hand, antimicrobial effectiveness might be considerably greater in the presence of enhanced metabolic activity in the parasite. Alternatively, interference with host defences by the hormones might reduce the total effect produced by the chemotherapy and this might take the form of an increased tendency for the drug-controlled infection to relapse after cessation of therapy.

A study has therefore been made of populations of tubercle bacilli in mice receiving cortisone or corticotrophin in various combinations with four chemotherapeutic agents, and the results are reported here.

MATERIALS AND METHODS

The methods and materials used were essentially the same as those described in the previous paper.

Albino mice infected intravenously with *Mycobacterium tuberculosis hominis* (H37Rv) were divided into groups according to the drugs used. Administration of all drugs and hormones was started on the day of infection, and was continued throughout the entire 8-week period of each experiment. Three to 5 animals were killed at intervals and the microbial populations in the lungs and spleens were determined as already described.

Sensitivity of the method

In the experiments concerned with the conventional antituberculous drugs the animal tissues were homogenized in 5 ml. saline containing 2 per cent bovine albumin. Three 0·02 ml. drops of appropriate dilutions of the homogenates were cultivated on oleic acid albumin agar from which colony counts were made after 2 and 4 weeks of incubation. Taking into account the size of the organ and various dilution factors it was theoretically possible to detect 70–90 cultivable units of tubercle bacilli present in either lung or spleen. In the experiments in

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which pyrazinamide was used it was necessary to increase the sensitivity of the method because of the marked antimicrobial effect of the drug combinations containing pyrazinamide (McCune, Tompsett and McDermott, 1956). To do this, the amount of the albumin-saline diluent used for suspending the tissues was reduced to 2·5 ml. and as much of a homogenate as possible was cultivated on a plate. With this modification of the technique it was possible in theory, to detect 1 to 3 cultivable units in either the lung or the spleen.

Preparations used

Streptomycin sulphate 4 mg. given once daily intramuscularly, equivalent to 200 mg./kg./day. Para-amino-salicylic acid 0·75 per cent in diet daily, equivalent to 1·5 g./kg./day. Isoniazid 0·0125 per cent in diet daily, equivalent to 25 mg./day. Pyrazinamide 2·0 per cent in diet daily, equivalent to 4 g./kg./day. Cortisone acetate (Schering) 0·1 mg. in aqueous crystalline suspension, 0·1 ml. daily intramuscularly. Corticotrophin (Acthar Gel, Armour) 2·0 mg. in 0·1 ml. of hydrolysed gelatin twice daily intramuscularly.

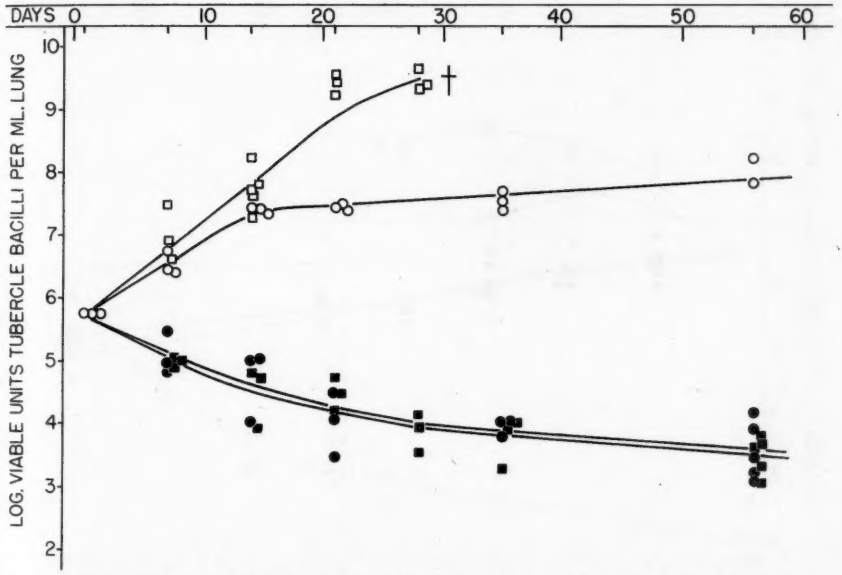

FIG. 1.—Influence of corticotrophin and streptomycin-PAS used singly and together on microbial populations (H37Rv) in mouse lungs during 56 days of therapy. Infecting inoculum: $3 \cdot 3 \times 10^6$ cultivable units tubercle bacilli. No animals treated with corticotrophin alone survived after 28 days.

○———○ control.
●———● streptomycin and PAS.
□———□ corticotrophin.
■———■ ,, , streptomycin and PAS.

When the drug was given in the diet, it was intimately mixed with finely ground pellets in a McLelland dry batch mixer. Previous measurements of food consumption and body weight of animals treated according to this method have shown that drug intake remains constant (McCune and Tompsett, 1956).

The results are presented either in graphic form similar to that used in the preceding paper or in Tables. Each symbol in the graphs indicates the logarithmic value of the number of " cultivable units " of *Myco. tuberculosis* per unit volume of the lungs or spleen of one

animal. Trend lines were then drawn with reference to the average logarithmic value of each group. The numbers of animals in each group at each point of time varied from 3 to 5.

The variation in the number of animals was caused both by premature deaths and, in the early stages of the study, by the overgrowth of the indigenous microbial flora in some of the cultures. This overgrowth was observed most frequently in cultures from animals receiving hormones and drugs which acted only on tubercle bacilli. It did not occur in animals which received streptomycin. The non-tuberculous flora which emerged consisted of a wide variety of organisms which were predominantly Gram-negative. It was found that the emergence of these bacteria could be readily controlled by the addition of polymyxin at a concentration of 1000 units/ml. to the tissue homogenates. Simultaneous cultures without polymyxin showed that this substance did not inhibit the growth of *Myco. tuberculosis* in these circumstances.

Observations were also made on the nature of the tissue changes and on the survival of the animals in the various experimental groups.

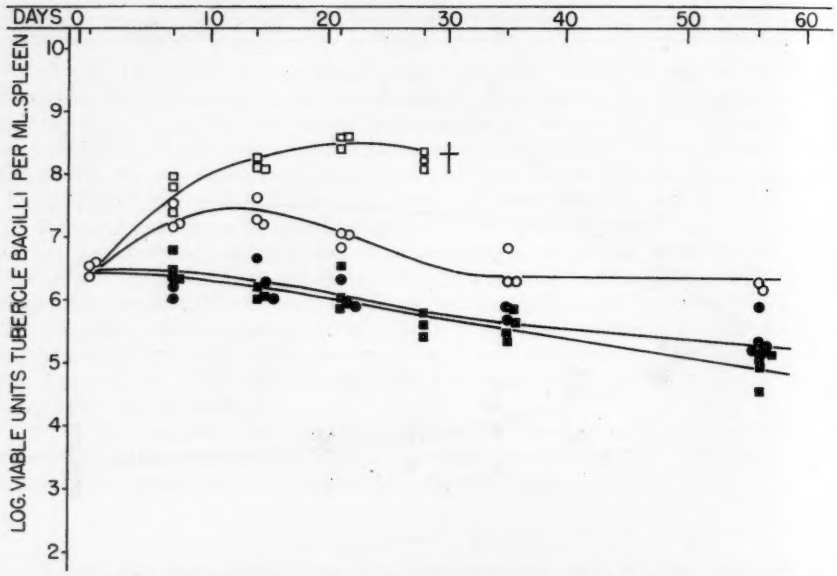

FIG. 2.—Influence of corticotrophin and streptomycin-PAS used singly and together on populations of *Myco. tuberculosis* (H37Rv) in spleens of the same animals whose lung populations are shown in Fig. 1.

○——○ control.
●——● streptomycin and PAS.
□——□ corticotrophin.
■——■ ,, , streptomycin and PAS.

RESULTS

Streptomycin and Para-amino-salicylic Acid (PAS)

In Fig. 1 and 2 are shown the trend lines of the populations of tubercle bacilli in the lungs and spleens of the mice which received streptomycin-PAS and corticotrophin, given alone or in combination. Similar results were obtained with cortisone and these are given in Table I.

TABLE I.—*Log_{10} Viable Units* of Tubercle Bacilli (H37Rv) per ml. of Tissue in Animals Treated with Cortisone and Streptomycin-PAS, Alone and in Combination*

Tissue	Treatment group	Duration of infection in days					
		1	7	14	21	35	56
Lung	Control	5·7	6·5	7·4	7·5	7·6	8·0
	Cortisone	—	6·5	7·5	8·1	8·6	9·0
	Cortisone, streptomycin-PAS	—	4·8	4·6	4·3	4·2	3·9
	Streptomycin-PAS	—	5·1	4·8	4·2	4·0	3·6
Spleen	Control	6·5	7·3	7·4	7·0	6·5	6·2
	Cortisone	—	7·6	7·8	7·6	7·4	7·6
	Cortisone, streptomycin-PAS	—	6·3	6·0	6·1	5·5	5·4
	Streptomycin-PAS	—	6·2	6·3	6·0	5·7	5·4

* Mean of the values obtained from 3–5 animals.

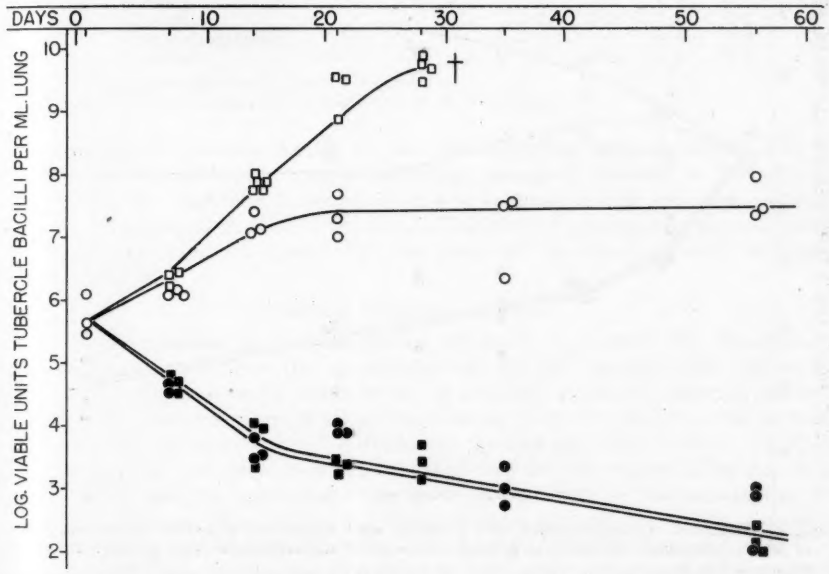

FIG. 3.—Influence of corticotrophin and isoniazid used singly and together on microbial populations (H37Rv) in mouse lungs during 56 days of therapy. Infecting inoculum: $3·2 \times 10^6$ cultivable units tubercle bacilli.

○——○ control.
●——● isoniazid.
□——□ corticotrophin.
■——■ ,, + isoniazid.

The trends of the populations of tubercle bacilli in the untreated control mice and in the animals which received streptomycin-PAS are similar to those previously reported (McCune and Tompsett, 1956). In the lung, the population underwent a considerable reduction during the first 3 weeks of streptomycin-PAS therapy but the census fell only slightly thereafter. In the spleen, the initial multiplication of bacilli that has been shown to occur when streptomycin is used alone was

prevented by administration of streptomycin-PAS. The populations then stabilized and persisted at a significantly lower level than in the untreated control animals. The behaviour of the populations of tubercle bacilli in the animals maintained under the influence of cortisone or corticotrophin without other treatment was the same as described in the previous paper.

Streptomycin-PAS was as effective in reducing, or stabilizing, the populations of tubercle bacilli in the animals maintained on either hormone as was the case in the animals which received no hormone.

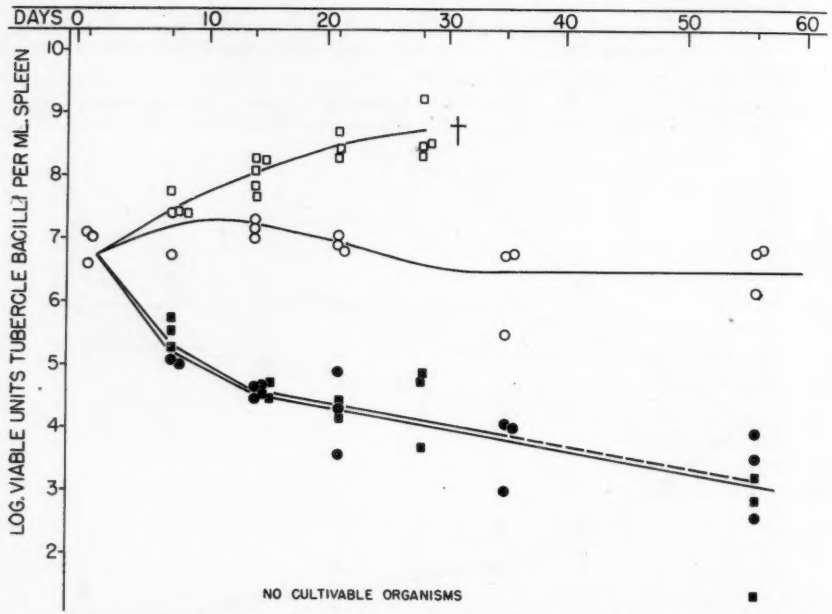

FIG. 4.—Influence of corticotrophin and isoniazid used singly and together on populations of *Myco. tuberculosis* (H37Rv) in spleens of the same animals whose lung populations are shown in Fig. 3.

Isoniazid

The results of the experiments with the two hormones and with isoniazid may be seen in Fig. 3 and 4 and Table II. As previously reported (McCune and Tompsett, 1956), isoniazid alone effected a considerable reduction in the populations in both the spleen and lungs and this reduction was considerably greater than that which occurred with streptomycin-PAS. In the spleen, the number of bacilli fell to about 1000/ml. of tissue, while in the lung there was a reduction to about 500/ml. Tubercle bacilli were recovered from all animals at all stages of the 8-week period of isoniazid therapy. In similar experiments it was demon-

strated by drug-susceptibility studies *in vitro* that the persistence of the populations in the latter phases of the treatment period bears no relation to the proportion of drug-resistant mutants (McCune and Tompsett, 1956).

TABLE II.—*Log_{10} Viable Units* of Tubercle Bacilli (H37Rv) per ml. of Tissue in Animals Treated with Cortisone and Isoniazid, Alone and in Combination*

Tissue	Treatment group	Duration of infection in days					
		1	7	14	21	35	56
Lung	Control	5·7	6·1	7·2	7·3	7·2	7·6
	Cortisone	—	6·4	7·7	8·5	8·7	8·5
	Cortisone-isoniazid	—	4·4	3·6	3·6	3·2	2·5
	Isoniazid	—	4·6	3·5	3·9	3·0	2·7
Spleen	Control	6·9	7·1	7·2	6·9	6·3	6·6
	Cortisone	—	7·1	7·9	7·6	7·4	7·5
	Cortisone-isoniazid	—	5·2	4·3	4·4	4·5	3·6†
	Isoniazid	—	5·0	4·6	4·2	3·7	3·3

* Mean of the values obtained from 3–5 animals.
† No organisms were obtained from the spleen in the one animal.

The census of tubercle bacilli in the tissues of the animals maintained on cortisone or corticotrophin while receiving isoniazid showed a fall closely corresponding to that which occurred in the animals which received isoniazid alone. In one animal in each of the isoniazid-hormone groups, no tubercle bacilli were cultivated from the spleen, but the lungs of the same animals contained cultivable bacilli.

Pyrazinamide and Isoniazid

In previous studies in this laboratory (McCune, Tompsett and McDermott, 1956), it was shown that the administration of the nicotinamide derivative pyrazinamide, together with isoniazid in appropriate doses, to tuberculous mice resulted in the disappearance of all tubercle bacilli from the tissues so far as could be determined by microscopy, culture, or guinea-pig inoculation. In these circumstances the infection is not eliminated but is only rendered latent, since tubercle bacilli can be cultivated from at least one-third of the animals in the experimental groups when left for a further 90 days without treatment, extensive sampling having previously revealed no bacilli. This "disappearance" of tubercle bacilli from the tissues occurs only with drug combinations containing pyrazinamide.

Because of the extremely high degree of effectiveness of the pyrazinamide-isoniazid regimen on tubercle bacilli in mice, its use in animals also treated with cortisone or corticotrophin was investigated. The more sensitive method for cultivating the tubercle bacilli in the tissues was used in these experiments (see Materials and Methods).

Influence of hormones on effectiveness of pyrazinamide-isoniazid as measured by disappearance of tubercle bacilli during drug administration

The results of these experiments are presented in Fig. 5–8. In the animals maintained on either of the two hormones and treated with pyrazinamide-isoniazid, no tubercle bacilli could be cultivated from the lung after the third week of therapy. The splenic populations of tubercle bacilli likewise fell during

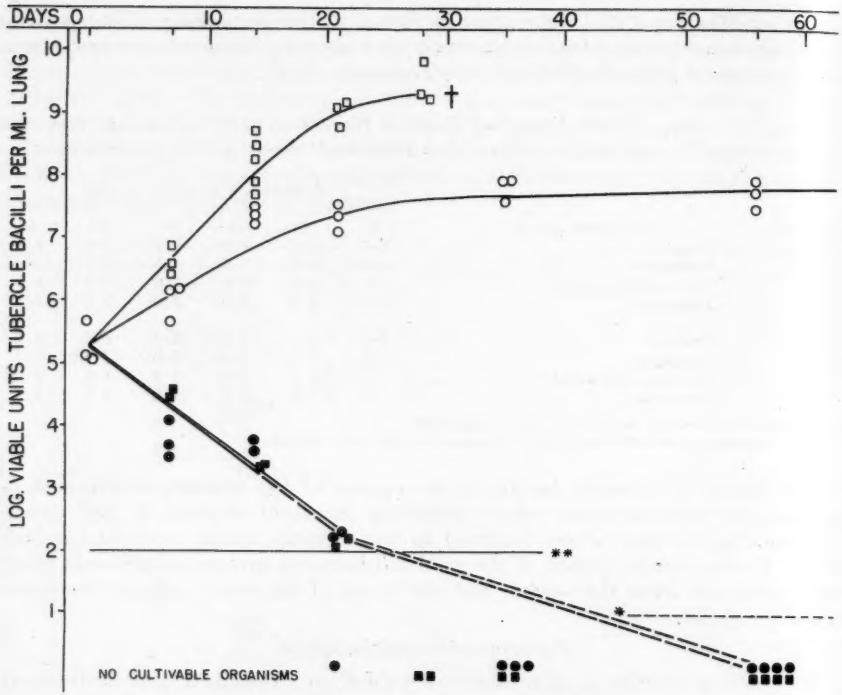

FIG. 5.—Influence of corticotrophin and pyrazinamide-isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs. Infecting inoculum: $3 \cdot 2 \times 10^6$ cultivable units tubercle bacilli.

○——○ control.
●——● pyrazinamide and isoniazid.
□——□ corticotrophin.
■——■ ,, , pyrazinamide and isoniazid.

† The method used during the first 35 days of the experiment permitted detection of 70–90 cultivable units of tubercle bacilli per lung.

* The method used on the 56th day permitted detection of 1–3 cultivable units of tubercle bacilli per lung.

pyrazinamide-isoniazid therapy both in the animals maintained on either of the two hormones and in the animals which received no hormone. At the completion of the 8-week experimental period, tubercle bacilli could be cultivated from the spleens of only two animals each of which had received one or two of the hormones.

In the previously cited studies with pyrazinamide-isoniazid, it was found that 8 weeks of pyrazinamide administration in a total period of 12 weeks of continuous chemotherapy were necessary to produce the complete "disappearance" of tubercle bacilli with regularity. Consequently the present experiments, in which the total period of therapy was only 8 weeks, provide no evidence that the full effectiveness of the antimicrobial therapy was lowered in the animals which received cortisone or corticotrophin.

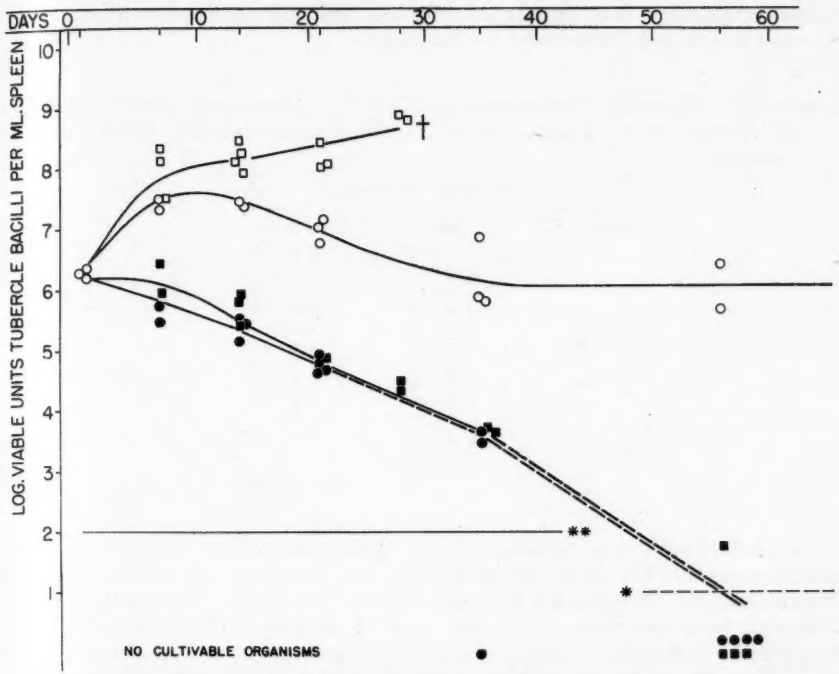

FIG. 6.—Influence of corticotrophin and pyrazinamide-isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung populations are shown in Fig. 5.

○——○ control.
●——● pyrazinamide and isoniazid.
□——□ corticotrophin.
■——■ ,, , pyrazinamide and isoniazid.

† The method used during the first 35 days of the experiment permitted detection of 70–90 cultivable units of tubercle bacilli per lung.

* The method used on the 56th day permitted detection of 1–3 cultivable units of tubercle bacilli per lung.

Influence of hormones on effectiveness of pyrazinamide-isoniazid as measured by incidence of post-treatment reappearance of tubercle bacilli

In an additional experiment, 22 mice were treated for 8 weeks with pyrazinamide, isoniazid, and cortisone and were then observed for a further 16-week period during which they received neither hormone nor antimicrobial therapy. At the end of the treatment-free period, the surviving animals were killed and the spleen and lungs were cultivated. The results are presented in Table III.

One animal died during chemotherapy 9 weeks after infection and no tubercle bacilli were detected in either organ. Another died 5 weeks after the completion of treatment, and approximately 400 cultivable units of tubercle bacilli were found in the lungs and 200 in the spleen. Four others also died during the latter 12 weeks of the experiment, but were autolysed to such an extent that bacterio-

logical examination was impossible. Sixteen animals survived until the end of the experiment and 2 were used for histology.

TABLE III.—*Microbial Populations (H37Rv) in Spleens and Lungs of* 14 *Mice* 16 *Weeks after Discontinuing Pyrazinamide-Isoniazid Treatment with Cortisone*

	8 weeks' treatment	
Animal No.	Spleen	Lung
1	$1{\cdot}1\times10^{6}$	$2{\cdot}2\times10^{2}$
2	$2{\cdot}5\times10^{5}$	6×10^{0}
3	$2{\cdot}5\times10^{5}$	3×10^{0}
4	$2{\cdot}3\times10^{5}$	$3{\cdot}0\times10^{3}$
5	$1{\cdot}7\times10^{5}$	0
6	$1{\cdot}7\times10^{5}$	$4{\cdot}5\times10^{4}$
7	$1{\cdot}6\times10^{5}$	$1{\cdot}3\times10^{3}$
8	$1{\cdot}2\times10^{5}$	$1{\cdot}8\times10^{4}$
9	$1{\cdot}1\times10^{5}$	$1{\cdot}0\times10^{1}$
10	$2{\cdot}7\times10^{4}$	0
11	$9{\cdot}0\times10^{3}$	0
12	$3{\cdot}8\times10^{3}$	0
13	$8{\cdot}0\times10^{2}$	3×10^{0}
14	0	0

Tubercle bacilli were cultivated from the spleens of 13 of the 14 surviving animals available for study 16 weeks after the completion of an 8-week period of administration of cortisone, pyrazinamide and isoniazid. The bacilli were also cultivated from the lungs of 9 of the same 14 animals. This incidence of reappearance of cultivable tubercle bacilli after treatment in the animals which received cortisone during the period of pyrazinamide-isoniazid therapy is appreciably higher than has been previously observed with pyrazinamide-isoniazid in animals which received no hormone. In the last-named experiments, however, the antituberculous drugs had been given for 12 weeks.

Pathology

Animals which died during the experimental period

No evidence of tuberculosis was discovered in the 17 animals that received hormone and antituberculous chemotherapy and died during the course of the experiments. Four of the deaths occurred immediately after injections of streptomycin and the animals showed evidence of a state resembling shock. Five deaths occurred within one week in a group of corticotrophin-treated animals housed in the same cage. The animals all developed diarrhoea and at autopsy no evidence of tuberculosis was discovered. It was expected that deaths would occur during the administration of these hormones as a result of superinfection by the indigenous flora of the animals. The absence of deaths in the animals receiving streptomycin, except for the traumatic deaths mentioned above, makes it seem likely that such a mechanism was operative in the animals which died without evidence of significant tuberculous infection. Pulmonary lesions were observed in only one animal. There were 2 deaths in animals which received pyrazinamide-isoniazid without hormone administration.

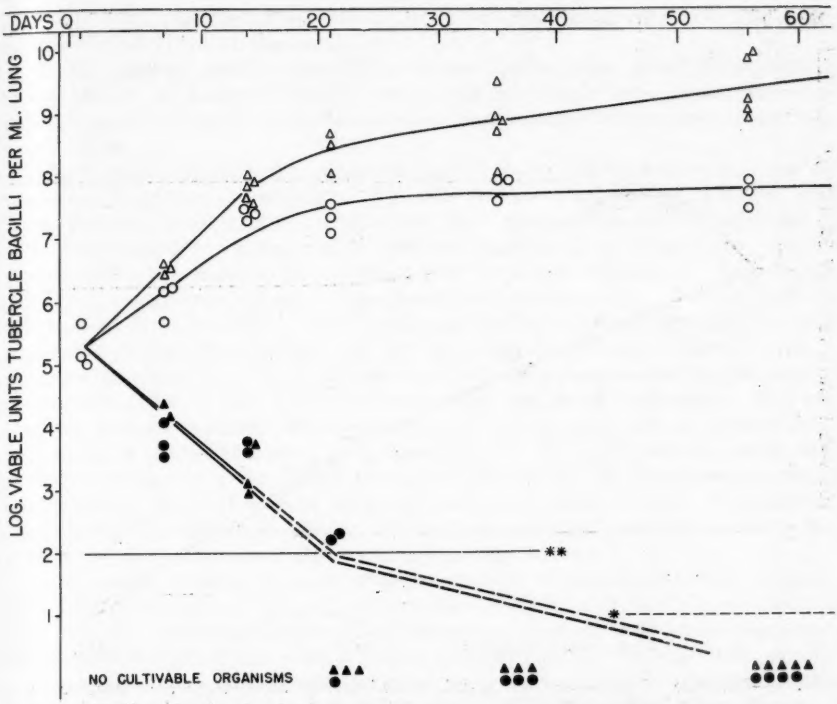

FIG. 7.—Influence of cortisone and pyrazinamide-isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs. Infecting inoculum: $3 \cdot 2 \times 10^6$ cultivable units tubercle bacilli.

○——○ control.
●——● pyrazinamide and isoniazid.
△——△ cortisone.
▲——▲ „ , pyrazinamide and isoniazid.

† The method used during the first 35 days of the experiment permitted detection of 70–90 cultivable units of tubercle bacilli per lung.
* The method used on the 56th day permitted detection of 1–3 cultivable units of tubercle bacilli per lung.

Animals killed at selected periods

The gross and microscopic changes which occurred in the infected untreated animals and those receiving corticotrophin or cortisone were described in the previous paper. The drug-treated animals which received either of the hormones were smaller than those which received no hormone, and showed the same changes in the adrenal, spleen and thymus as described in the previous paper for the animals which received hormone but no antituberculous therapy.

There were definite histological changes in the lungs of animals which received antimicrobial treatment without hormone. These changes could not be differentiated from those present in the animals which received any of the three drug regimens together with either hormone. The tissue changes consisted of scanty

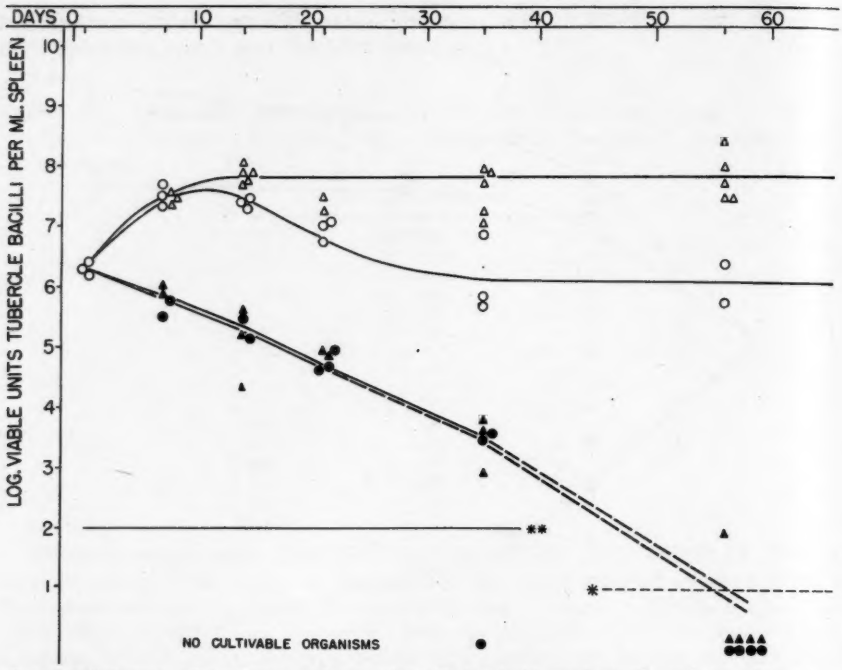

FIG. 8.—Influence of cortisone and pyrazinamide-isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung populations are shown in Fig. 7.

○——○ control.
●——● pyrazinamide and isoniazid.
△——△ cortisone.
▲——▲ ,, , pyrazinamide and isoniazid.

† The method used during the first 35 days of the experiment permitted detection of 70–90 cultivable units of tubercle bacilli per lung.

* The method used on the 56th day permitted detection of 1–3 cultivable units of tubercle bacilli per lung.

mononuclear cell aggregations and some scattered infiltration of alveolar walls by the same cells. Some fatty macrophages were seen, but no necrosis was observed.

A few acid-fast organisms were seen in the sections of the lung of animals which had received streptomycin-PAS. No organisms were demonstrated in the animals which received streptomycin-PAS together with either hormone, nor indeed in any animal which received either of the other drug regimens after the third week of therapy.

DISCUSSION

On the basis of studies on microbial populations, supported by observations on survival and the tissue changes in albino mice infected with *Myco. tuberculosis* (H37Rv) it is concluded that the antimicrobial activities of streptomycin-PAS,

isoniazid, and pyrazinamide-isoniazid were not influenced by either cortisone or corticotrophin given at the same time.

In the present study cortisone or corticotrophin were given throughout the entire period of antimicrobial therapy and gave rise to a great increase in populations of tubercle bacilli in those animals which did not receive antituberculous drugs.

The fundamental nature of the actions of both the hormones and the antimicrobial drugs remains obscure. There is sufficient knowledge, however, of the properties of antimicrobial drugs and the phenomena associated with the administration of the hormones to theorize that the latter might alter the host-drug-parasite relationship in either of two opposing directions. An abundant growth of organisms occurs in the favourable environment created under the influence of the hormones ; this enhancement of metabolic activity of the micro-organisms might thus render the antimicrobial drugs more effective. In the experiments cited, however, treatment and infection were started on the same day so that it is unlikely that bacillary metabolism was greatly increased. Nevertheless, the micro-organisms were subsisting in an environment so altered by the hormones that in the absence of drug therapy, their metabolic activity, as expressed in this multiplication, would have been at its maximum. Experiments are planned to determine the influence of these antimicrobial substances on populations of tubercle bacilli which have already attained the maximal metabolic activity made possible by the influence of the hormones on the host.

The results reported in these experiments are of interest therefore in that all three drug regimens, namely streptomycin-PAS, isoniazid, and isoniazid-pyrazinamide, maintained their effectiveness when given with either cortisone or corticotrophin during the 8-week period of observation. On the other hand, no evidence of an *increased* antibacterial activity was obtained. In the hormone-influenced animals isoniazid or streptomycin-PAS did not produce the "vanishing" phenomenon customarily produced by pyrazinamide-isoniazid. Instead the microbial populations in the lungs and spleens of animals receiving streptomycin-PAS or isoniazid become stabilized after initial reduction ; any change whether in the form of an increase or decrease in the populations specified, under additional hormonal influence would be readily discernible. In these experiments it was noted that these particular populations continued to remain stabilized during hormone administration, thus providing suggestive evidence that antimicrobial effectiveness was neither enhanced nor impaired.

An equally sensitive index existed in those animals receiving pyrazinamide-isoniazid in which the numbers of tubercle bacilli in the spleen were reduced to a level below which they could not be detected. Even when the technique had been adjusted to permit detection of 1–3 organisms per spleen, neither hormone interfered with the unique action of this drug combination. Tubercle bacilli reappeared in animals treated with pyrazinamide-isoniazid for two months and subsequently left untreated for a further four months. This is not necessarily an indication that the antimicrobial activity was prejudiced by hormonal influence, because it has been repeatedly observed in other experiments (McCune, *et al.*, 1956). Nevertheless, the fact that the infection did relapse in the customary fashion provides no indication that the antimicrobial influence of pyrazinamide-isoniazid was increased by cortisone. Indeed, the high incidence of relapse in these experiments suggests that the host mechanisms for holding infection in check, after cessation

of antimicrobial drug therapy and the hormones, might have been impaired. This point was not clearly established in the present studies, however, because the total period of chemotherapy was only 8 weeks instead of the customary 12-week period used to produce the pyrazinamide-isoniazid phenomenon.

In previous studies on the use of antimicrobial drugs and steroids in experimental infections, minimally effective doses of the drugs have usually been employed and under these conditions it has been observed that the hormones render antimicrobial agents less active (Jawetz and Merrill, 1953). In experimental tuberculosis, most studies have been concerned with the effect of streptomycin alone and most observers arrived at similar conclusions (Karlson and Gaimer, 1951). Nevertheless, neither cortisone (1 mg. per day) nor corticotrophin (20 units per day) were found to have adverse effect on streptomycin activity in the rabbit (Morgan, Wanzer and Smith, 1954).

In man there are many reports of the exacerbation and reactivation of tuberculosis after the administration of corticotrophin or cortisone. In one report (American Trudeau Society, 1952) concerning 81 patients, it was reported that the infection in 12 of 25 patients receiving corticotrophin and 26 of 41 patients receiving cortisone was worse as a result of administration of the hormones. There is accumulating evidence, however, of the rarity of exacerbation of tuberculosis in man so long as *effective* antibacterial treatment is given with the hormone (Houghton, 1952). The use of cortisone and corticotrophin is now advocated in the treatment of late cases of tuberculous meningitis, hypersensitivity states occurring in the course of antimicrobial drug therapy (where no alternative treatment is possible) and in cases of fulminating tuberculosis. Other less pressing indications have been suggested. The place of these hormones, however, in the treatment of clinical tuberculosis remains *sub judice*.

The results of the experiments reported are considered to be in accord with the clinical experiments so far reported in man. They lend support to the use of cortisone and corticotrophin when there is some clear indication in tuberculous patients as long as effective antimicrobial treatment is given as well and continued for some time after the hormones have been stopped.

SUMMARY

A study is reported of the influence of cortisone and corticotrophin on the populations of human tubercle bacilli (H37Rv) in the lungs and spleens of albino mice which were simultaneously treated with streptomycin-PAS, isoniazid or isoniazid-pyrazinamide.

It is concluded on the basis of data presented, supported by observations on pathology and survival that antituberculous drug activity was neither enhanced nor diminished by the administration of cortisone or corticotrophin.

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THE EFFECTS OF HIGH BLOOD CHOLESTEROL ON THE PULMONARY ARTERIAL CHANGES PRODUCED IN RABBITS BY THE INJECTION OF BLOOD CLOT

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THE rôle of thrombosis in the production of atherosclerosis has been emphasized by Duguid (1946, 1948) but although the fibrotic element of atherosclerosis can be explained on this basis there is little good evidence at the moment to show that the lipid component has a similar origin. Harrison (1948) produced lesions in the pulmonary arteries of rabbits by the injection of finely fragmented human blood clot, which he considered indistinguishable from spontaneous arteriosclerosis. Because of the infrequent finding of lipid in the lesions he chose to use the term arteriosclerosis rather than atherosclerosis. Similarly Wartman, Jennings and Hudson (1951) failed to find lipid in lesions produced by a similar method using rabbit blood instead of human blood as a source of clot. It was therefore decided to inject rabbits with clots derived from the blood of rabbits with a high level of circulating cholesterol, to see if any cholesterol could be found in the organizing lesions in the pulmonary arteries. A further group of rabbits fed on a diet containing cholesterol was also injected with similar clots to see if the high blood cholesterol level of the recipients had any effect on the lesions produced. It was found that stainable lipid was present only rarely in the first group while in the second group it was present with great frequency. A striking feature arising out of the experiment was the finding of large amounts of lipid in the intima of pulmonary arteries of the rabbits with high blood cholesterol receiving emboli. This change is identical with the usual type of cholesterol-induced atheroma.

METHODS

Sixty-eight male Chinchilla rabbits, 6–8 months old, weighing approximately 2·5 kg. were used for the experiment. Four served as a source of cholesterol-rich blood from which emboli were prepared. These rabbits were fed on unlimited amounts of cholesterol-impregnated food prepared in a way described previously (Heptinstall and Bronte-Stewart, 1954). Whenever a batch of animals was due for injection of emboli, blood was taken from the marginal ear vein of one of the 4 donor animals under aseptic conditions and taken up into the stem part of a Pasteur pipette, freshly made from soda glass. Small emboli were prepared by the method described by Pryce, Pike and Gorrill (1950), which briefly consists of allowing the blood to clot in the pipette and expelling the " worm " so produced into a sterile Petri dish containing isotonic saline. The " worms " were then cut into small lengths with cutting needles so that the resultant emboli were cylinders approximately 1 mm. diameter and 1–2 mm. long, each injected animal receiving emboli equivalent to a 30 cm. length of " worm ". Injections of this amount of emboli suspended in 3 ml. of sterile isotonic saline were made into the marginal ear vein of the experimental rabbits through an 18 gauge needle on each of 3 consecutive days. Cholesterol estimations were made on the blood from which the emboli

were prepared by the method of Pearson, Stern and McGavack (1953), each time the animals were bled.

The experimental rabbits were divided into 4 groups.

Group I consisted of 24 rabbits, fed on diet 18 of Bruce and Parkes (1946), hay and unlimited water. They were injected as described above on each of 3 consecutive days, and were killed at intervals of between 3 and 21 days, the time being reckoned from the day of the first injection.

Group II consisted of 20 rabbits which had been fed on a diet containing 0·75 per cent cholesterol for 10 days, each animal consuming approximately 0·6 g. of cholesterol per day. On the 10th day of feeding, blood was taken for cholesterol estimation and 3 injections of emboli made on consecutive days as in Group I. Cholesterol feeding was continued during the injection days and stopped after a total of 17 days' feeding, when a further cholesterol estimation was made. Those rabbits allowed to survive after this time were given a normal diet for the remainder of their lives and a final cholesterol estimation was made on the day of death. Animals were killed at intervals of 3–21 days from the day of the first injection.

Group III was made up of 10 rabbits to serve as controls and they received no injections and were fed on a normal diet as in Group I.

Group IV was a further control group consisting of 10 rabbits which received no injections but which were fed on a cholesterol-containing diet as in Group II for 17 days. Half were killed at this time and half allowed to live for one more week on a normal diet. Cholesterol estimations were made on the blood at 17 days in all rabbits and on the 24th day in those killed on that day.

All rabbits were killed by an overdose of intravenous Nembutal and post mortem the lungs were removed with a length of trachea. The lungs were injected with 10 per cent formol saline through the trachea, so that their final volume approximated to that during life. After fixation in a large volume of 10 per cent formol saline for 48 hours, thin slices were taken from the lower lobes of both lungs, cuts being made at right angles to the axial stem. Two slices from each lower lobe were processed for paraffin sections and slides were stained with haematoxylin-eosin, haematoxylin and van Gieson's mixture and for elastica using the Hart-Sheridan method. Frozen sections were prepared from one slice from each lower lobe and stained with Sudan III.

RESULTS

The mean serum total cholesterol value for the blood samples from which clots were made was 1900 $\pm$ 700 mg./100 ml.

All animals except 3 which died suddenly following the final injection tolerated the injections well, and remained healthy until they were killed.

Group I

In rabbits killed or dying within 7 days of the first injection there was a variable degree of acute arteritis affecting pulmonary arteries of all sizes. This consisted of infiltration of the intima, adventitia and sometimes media by polymorphs and mononuclear cells, and proliferation of the endothelial cells. The intima was sometimes oedematous with apparent splitting of elastic fibres. The reaction was most intense in areas where emboli had become impacted (Fig. 1) but was also seen in the apparent absence of emboli (Fig. 2). In rabbits killed between 8 and 14 days the arteritis was less severe and in those killed at 21 days no arteritis was found.

There was evidence of organizing emboli in 9 out of 12 rabbits killed by the 7th day. It took the form of areas of fibrin either invested or becoming invested by endothelium and being invaded by large numbers of polymorphs and mononuclears (Fig. 3). In only 2 out of 12 rabbits killed at this stage was there evidence of anisotropic lipid in the emboli and this was contained in small numbers of foam cells. In lesions of over 7 days the emboli were less cellular, devoid of fibrin

and contained young collagen. Evidence of cholesterol in organizing emboli was present in only 1 of the 7 rabbits with lesions of 8–14 days' duration. At 21 days organizing emboli were no longer seen.

In no less than 22 rabbits there was evidence of fibro-elastic thickening of the intima of pulmonary arteries, of varying degrees of severity. This usually took the form of eccentrically arranged plaques, slightly raised above the surface and containing numerous layers of elastic fibres and varying amounts of collagen (Fig. 4). This type of lesion was found in particular at places where the thickness of the vessel wall became reduced to give rise to thinner-walled branches. The thickenings were found to bear no relationship to the time between injections and death, but in rabbits with the longer intervals there was frequently a diffuse reduplication of the internal elastic lamina.

A foreign body giant cell reaction to cotton thread, presumably derived from cotton wool used to sterilize the ear before injection, was found in the artery walls in several rabbits.

Group II

Arteritis of similar frequency and intensity was found in animals killed within 7 days of injection. One big difference however was that mononuclear cells containing anisotropic material were frequently found in the intima of affected vessels.

Emboli took the same form as in Group I except that in many cases cholesterol-containing mononuclear cells were present in varying numbers in the organizing lesion (Fig. 5). In addition, polypoidal masses of foam cells were present, arising from the intima and projecting into the lumen (Fig. 6 and 7). It is unlikely that these were emboli because of the absence of fibrin in those killed before 7 days, and also because similar lesions are found in rabbits, not receiving emboli, which are fed on cholesterol for long periods of time. The numbers of emboli found in this group were roughly the same as in Group I.

An additional feature was the presence of large numbers of foam cells containing anisotropic material in the intima of the pulmonary arteries in most of the rabbits. Sometimes this was a diffuse change affecting the whole of the circumference of the vessel with foam cells either one or several layers thick (Fig. 8 and 9). Often however the collections affected only a part of the circumference and as described before were sometimes polypoidal. Areas of intimal fibro-elastic thickening were found as in Group I (Fig. 10), but some of these areas sometimes contained foam cells which still further accentuated the splitting of the elastica. This gave the impression, probably false, that the lesions of this type were more common in this group than in Group I.

The peak serum cholesterol levels and amount of cholesterol laid down in pulmonary arteries are shown in Table I.

Group III

Arteritis was absent in all animals except one which showed a few polymorphs in the intima of one vessel. Mild degrees of eccentric fibro-elastic thickening were present in 6 cases and a moderate degree in 2 (Fig. 11). As in previous groups the change was most common at places where the vessel wall became thinned (Fig. 12). No evidence of emboli and no lipid deposits were found.

TABLE I.—*Peak Serum Total Cholesterol Levels and Amount of Cholesterol Deposited in Intima of Pulmonary Arteries in Rabbits of Group II*

Number of days between first injection and death	Number of rabbit	Peak serum total cholesterol level (mg./100 ml.)	Degree of intimal foam cell infiltration
3–7	143	1700	++
	128	1900	+
	113	1600	++++
	117	1350	+++
	118	1450	++++
	119	1200	++++
	123	1300	+++
	124	900	++
8–14	110	450	+
	111	800	+
	121	500	+++
	122	950	++
	127	1450	+++
	144	1200	++++
15–21	107	300	+
	109	850	+
	112	700	++++
	114	800	++
	125	1600	++
	126	250	++

0 No foam cells seen.
\+ Occasional foam cells in scattered arteries.
++ Moderate numbers of foam cells in scattered arteries or small numbers of foam cells in many arteries.
+++ Moderate numbers of foam cells in many arteries.
++++ Large numbers of foam cells in many arteries.

Group IV

Two animals showed mild endothelial proliferation of pulmonary arteries and one showed a few polymorphs in the intima of one vessel. Mild or moderate degrees of intimal fibro-elastic thickening were found in all but one rabbit and cholesterol was either absent or present in only small amounts in the intima of pulmonary vessels. This latter finding was in marked contrast to the lesions in animals of Group II.

The peak serum total cholesterol levels and amounts of cholesterol deposited in the pulmonary arteries are shown in Table II.

TABLE II.—*Peak Serum Total Cholesterol Levels and Amount of Cholesterol Deposited in Intima of Pulmonary Arteries in Rabbits of Group IV*

Number of rabbit	Peak serum total cholesterol level (mg./100 ml.)	Degree of intimal foam cell infiltration
135	700	+
136	2400	+
137	1350	+
138	1750	+
139	1750	+
140	1050	0
141	1700	+
142	1300	+
162	1400	+
163	1100	0

0 No foam cells seen.
\+ Occasional foam cells in scattered arteries.

DISCUSSION

In rabbits killed up to 14 days after injection, emboli in various stages of organization can be seen in both Groups I and II. There appears to be no difference in the incidence and mode of organization of emboli between the two groups, but a point of difference is the pronounced increase in cholesterol-containing macrophages (foam cells) in the emboli of Group II. Three rabbits from Group I did however have such cells present in the organizing emboli. Wartman *et al.* (1951) found no lipid in the lesions produced by injecting clots made from rabbits on a normal diet, and Harrison (1948) found it in only one rabbit out of ten, using human blood as a source of emboli. These observations suggest that lipid might only be found in organizing and organized emboli if either the recipient had a high level of circulating cholesterol or if the clots were prepared from blood with a high level of cholesterol. The level of total serum cholesterol of the donor blood was invariably over 1000 mg./100 ml., and in spite of this only 3 rabbits in Group I showed cholesterol in the lesions produced. However when the blood cholesterol in the recipient rabbit was high, as in Group II, the peak levels of serum total cholesterol ranging from 250 to 1900 mg./100 ml., then foam cells were found in the organizing emboli. It is difficult to understand the findings of Thomas, O'Neal and Lee (1956) who found lipid in 40 per cent of their rabbits receiving emboli prepared from rabbit blood. They suggested that the amount of red cells in their emboli was greater than that used by Wartman *et al.* (1951) and that the lipid came from the lipid capsules of the red blood cells.

The incidence of fibrous and fibro-elastic thickenings in the intima was not markedly different in Groups I and II although the presence of foam cells tended to accentuate the lesions in Group II. Thomas *et al.* (1956) found this type of lesion to be more prominent in a group of rabbits fed butter at the same time as emboli derived from rabbit blood were injected, than in a group receiving emboli

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EXPLANATION OF PLATES

FIG. 1.—Pulmonary artery with lodged embolus in lower part. Wall shows intense infiltration with polymorphs and mononuclears. Emboli injected 3 days before. Haematoxylin and eosin. × 100

FIG. 2.—Pulmonary artery with all coats infiltrated by polymorphs and mononuclears. No embolus in vessel. Emboli injected 3 days before. H. and E. × 120.

FIG. 3.—Organizing embolus showing cellular infiltration. Some cells are clear and contain lipid. Emboli injected 7 days before. Group I. H. and E. × 70.

FIG. 4.—Pulmonary artery with eccentric intimal thickening due mainly to elastic proliferation. Emboli injected only 2 days before. Elastic stain. × 85.

FIG. 5.—Pulmonary artery with organizing embolus. Embolus contains clear cells containing lipid. Emboli injected 14 days previously. Group II rabbit. H. and E. × 85.

FIG. 6.—Pulmonary artery with polypoidal collections of foam cells. Emboli injected 8 days previously in rabbit of Group II. H. and E. × 85.

FIG. 7.—Pulmonary artery with polypoidal collections of foam cells. Emboli injected 6 days before in rabbit of Group II. H. and E. × 85.

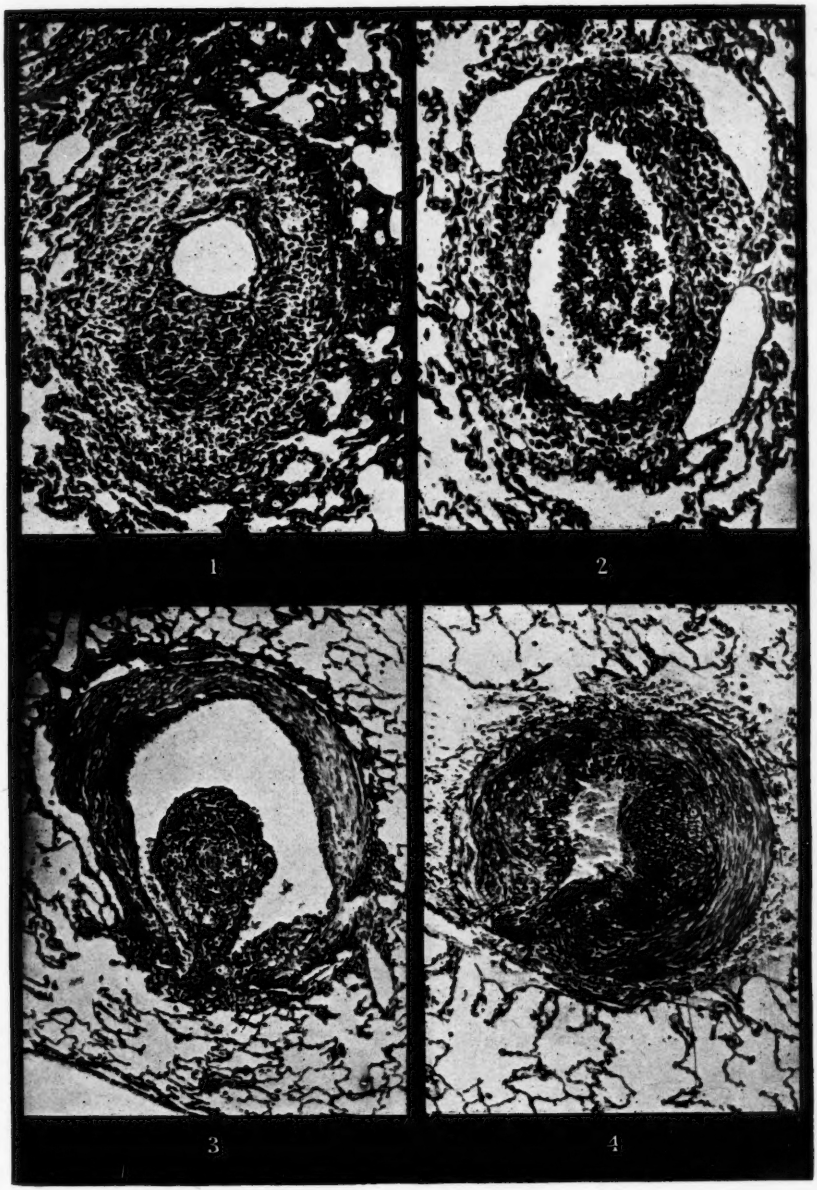
FIG. 8.—Pulmonary artery with thick layer of foam cells in intima. Emboli injected 8 days before into animal of Group II. Elastic stain. × 85.

FIG. 9.—Pulmonary artery with single layer of foam cells arranged around whole circumference. Emboli injected 8 days previously in a rabbit of Group II. Elastic stain. × 100.

FIG. 10.—Eccentric fibrous intimal thickening in pulmonary artery. Emboli injected only 4 days before. Elastic stain. × 120.

FIG. 11.—Artery showing eccentric elastic intimal thickening at place where vessel thickness changes and also at another site. Control animal. Elastic stain. × 85.

FIG. 12.—Artery showing intimal thickening at point of narrowing. Control animal. Elastic stain. × 110.



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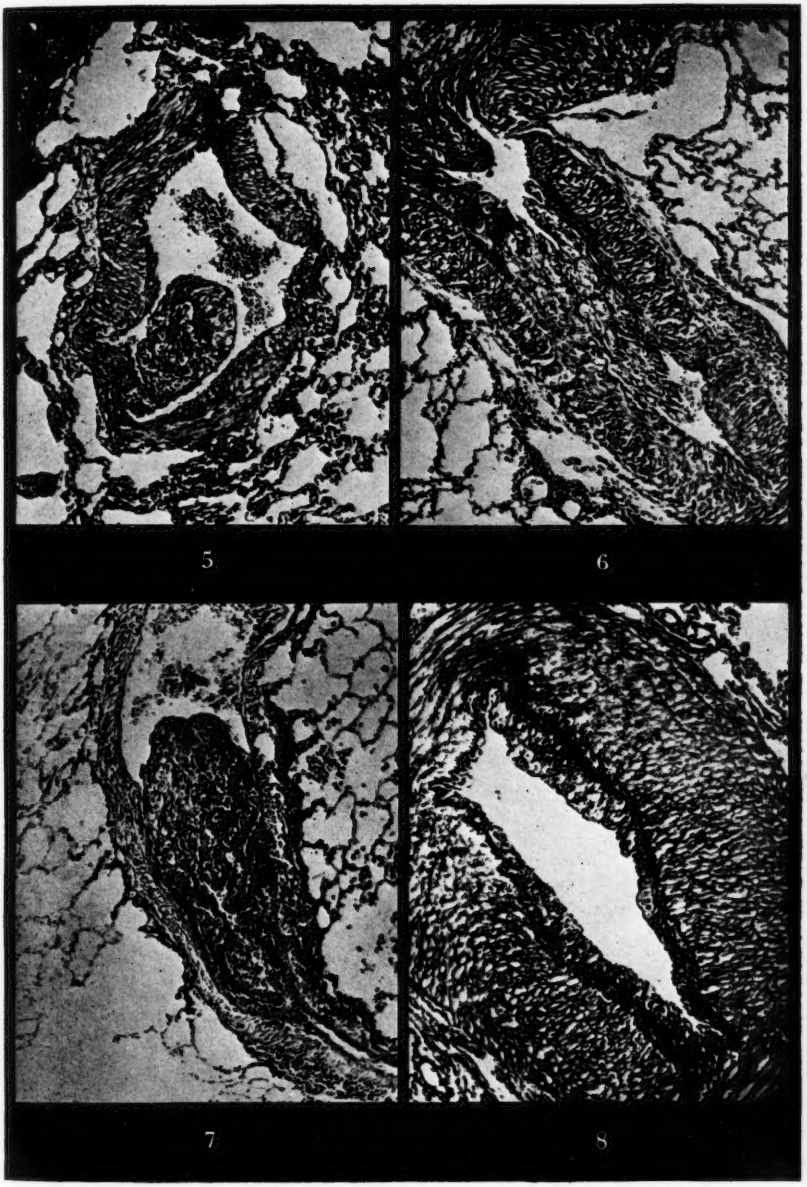

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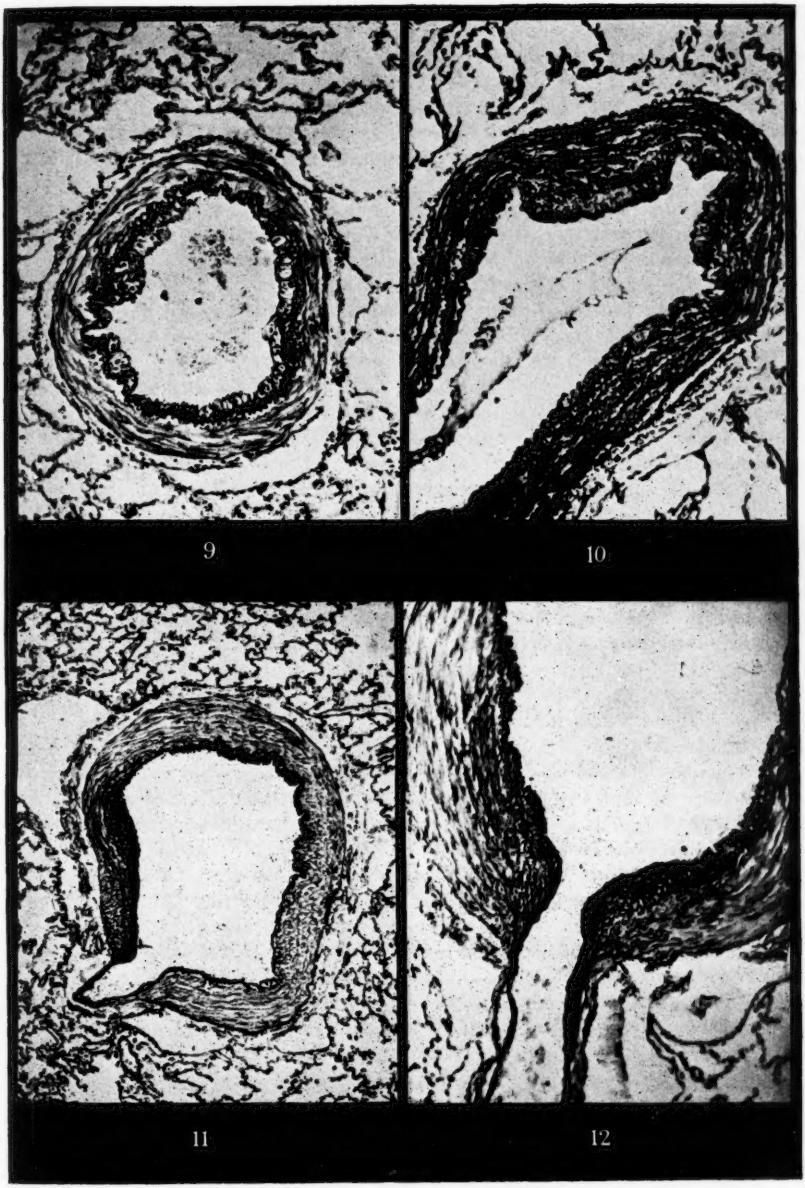

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only. The difference between their findings and those of the present experiment is possibly explicable on the basis that they used a complex lipid mixture in the form of butter while pure cholesterol was used in this experiment. Fibrinolysis is obviously a very potent force as shown by the relatively small numbers of emboli seen compared with the numbers injected, this having been previously noted by Heard (1952), and it is conceivable that the use of a complex lipid mixture might interfere with this. The presence of high blood cholesterol alone as in the present experiment seems to have little effect on fibrinolysis.

The number of lesions in the present experiment which could be accepted unequivocally as organized emboli, as opposed to the earlier organizing emboli, were few and were represented by raised patches of fibrous tissue containing small amounts of elastica. Other lesions consisting of similar plaques of fibro-elastic tissue or of elastic tissue with no fibrosis were more plentiful, and some at least of these lesions have in the past been accepted as organized emboli. However not only were these lesions found in rabbits injected with emboli only a few days before death, but in a high proportion of rabbits in the control Groups III and IV. The control rabbits like those of Groups I and II were 6–8 months old and of the same sex and strain. They had never at any time in their lives received any intravenous injections, except for the terminal intravenous Nembutal, and had been raised in hygienic surroundings and fed initially on bran and oats and latterly on diet 18 of Bruce and Parkes (1946). The most frequent site for such elastic and fibro-elastic thickenings was at places where the pulmonary arteries underwent abrupt changes in the thickness of the wall, this sudden thinning being a frequent finding in the rabbit lung. An alternative explanation of the sclerotic lesions in the pulmonary arteries of embolized rabbits was put forward by Muirhead and Montgomery (1951). They called attention to the intense arteritis found in such rabbits and suggested that this might be a factor in the production of the end picture. Barnard (1957) has produced similar lesions by the repeated intravenous injections of small amounts of air. Thus the finding of areas of fibro-elastic and elastic intimal thickening in the control rabbits of the present experiment must add a further note of caution in the interpretation of such lesions.

The most remarkable feature of this experiment however, is the large amount of cholesterol-containing macrophages in the intima of the pulmonary arteries of rabbits in Group II. This took the form either of focal plaques of foam cells or of a diffuse circumferential distribution of these cells, changes identical with those produced by the long-continued feeding of cholesterol. This is to be contrasted with the paucity of such collections in rabbits of Group IV, which received no emboli but which were fed on cholesterol for comparable periods to those in Group II. The amount of foam cells produced in the pulmonary arteries of Group II rabbits after approximately 3 weeks' cholesterol feeding was comparable with that obtained in non-embolized rabbits fed 0·5–0·6 g. cholesterol per day for 10 weeks, rabbits used in a previous experiment (Heptinstall and Bronte-Stewart, 1954) being used for comparison. The possible explanations are three. Firstly, that the transient pulmonary hypertension caused by embolization (Harrison, 1951) accentuates the amount of cholesterol deposited in the pulmonary arteries. High blood pressure in the major circulation greatly increases the amount of cholesterol-induced atheroma in rabbits (Bronte-Stewart and Heptinstall, 1954) and even short periods of high blood pressure have a similar though less pronounced effect (Heptinstall and Porter, 1957). This explanation

is considered unlikely since the duration of the increased pulmonary blood pressure is too short to account for such massive infiltration. Also the numbers of foam cells present is just the same in animals killed 8 days after embolization as in those with longer intervals between embolization and death, whereas a gradual build-up would be expected if hypertension were the cause. Secondly, it could be postulated that the increased foam cell deposition might be due to the organization of emboli into which had migrated large numbers of foam cells. This again is unlikely for the following reasons. The diffuse circumferential deposition is against such an explanation, for emboli are usually attached at one point of the circumference in an eccentric way. Again the foam cell deposits are great at sites where no emboli are present, in rabbits killed shortly after emboli have been injected, and therefore seen at a time before the emboli have organized. An additional factor to be considered is that even if an eccentrically arranged collection of foam cells is seen, it does not follow that this is due to emboli, for such collections are readily seen in rabbits fed on cholesterol for 10 weeks without emboli.

The third and most likely explanation is that the increased deposition is a consequence of the initial intense arteritis following injection of emboli. This type of arteritis has many of the features of the hypersensitivity type of arteritis and is present not only in vessels with emboli but also in pulmonary arteries away from the emboli. It is unlikely that this arteritis is a hypersensitivity phenomenon, since it appears within 24 hours of the first injection and in response to clot prepared from the same species. We can only speculate on the origin of such arteritis, but suffice it to say that it is a constant and very real change. It is suggested as an explanation of the large numbers of intimal foam cells in Group II rabbits that there is a migration of foam cells into the intima at the time of the acute arteritis, due to increased permeability of the intima, and that they remain there after resolution has occurred. The proliferation of endothelial cells may also play some part by taking on the rôle of phagocytes for cholesterol. A similar phenomenon has been described by Waters (1954) in the coronary arteries of dogs injured by the administration of allylamine, lipid being supplied at the same time in the form of intravenous egg yolk. Injury to the arterial wall has long been suggested as an explanation for the localization of atheromatous foci, and the findings in the present experiment fit well into this concept.

SUMMARY

Male Chinchilla rabbits were injected with blood clot prepared from rabbits with a high level of blood cholesterol and the pulmonary arteries examined at varying intervals of time. A further group of rabbits, fed on cholesterol for a total of 17 days was treated in a similar way.

Cholesterol was found in the organizing pulmonary emboli in only a small proportion of the first group but in a high proportion of the second.

Eccentric intimal fibro-elastic thickening of pulmonary arteries was found in both groups but was also found frequently in rabbits not receiving emboli.

The most remarkable finding was the tremendous number of cholesterol-containing macrophages (cholesterol-induced "atheroma") found in the pulmonary arteries of rabbits injected with clot while on a cholesterol-containing diet for only 17 days. The amount was roughly comparable to that found in non-embolized rabbits fed cholesterol in similar amounts for 70 days.

I am grateful to Miss Stella Hardie for her invaluable assistance during this experiment.

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ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION

III. FURTHER OBSERVATIONS ON THE PERMEABILITY FACTOR AND ITS INHIBITOR IN GUINEA-PIG SERUM

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THE main permeability factor (PF) in guinea-pig blood, and its inhibitor (IPF) can be studied either in native serum (Miles and Wilhelm, 1955) or in sera fractionated in aqueous ethyl-ether systems (Wilhelm, Miles and Mackay, 1955). In serum it appears on dilution in 0·85 per cent saline; after 1 hr. at room temperature, maximum activation occurs in a dilution of about 1/200 (PF/Dil) but not in less dilute serum owing to the presence of the IPF (the "dilution effect"). On fractionation of the serum, the PF is found as an α_2-globulin, the IPF as an α_1-globulin.

In this paper we report some further observations on the PF and IPF as manifested in serum dilutions, particularly with respect to the stability of the PF-IPF system in various experimental pathological states; and some refinements in the mode of isolation of PF from serum.

MATERIALS AND METHODS

As before, all our tests were made in adult albino guinea-pigs, and increased permeability measured as the area of exudation from the blood into the skin of the trunk of the vital dye pontamine blue (see Miles and Wilhelm, 1955). Methods not previously described are described below in their appropriate sections.

RESULTS

The Progressive Action of IPF in Serum Dilutions

The dilution effect

The typical curve of PF activity in serum dilutions 1–1½ hr. old (Fig. 1, A) summarizes our experience with several hundred sera from guinea-pigs. The maximum PF activity occurs at about 1/200 dilution. It declines in higher dilutions because the activity is diluted out, and in the lower dilutions because any PF activated during dilution is inhibited as the result of combination with IPF.

The curve A in Fig. 1 illustrates a feature we have not previously reported. The preferred way of harvesting serum is to defibrinate blood taken by heart puncture and immediately to remove the cells by centrifugation. Serum obtained

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in this way contains fewer traces of the minor PF that eventually develops (PF/Age), than serum obtained from the clot some 1½ hr. after bleeding the animal. These sera, nevertheless, with some constancy exhibited a slight secondary peak at a dilution of 1/5.

As the age of the dilutions increases beyond 1½ hr., the PF peak shifts to higher dilutions, moving to 1/1200 after 3 days at 4°. During the same period, the trough of inhibition widens by extending to higher dilutions (Fig. 1, Curve C); that is, the low concentration of IPF in the higher dilutions of serum, although sufficient to inhibit late activated PF, does so only after a time-lag, during which activation of the lower concentration of PF-precursor in more dilute serum takes place. The distance on the logarithmic scale of serum dilutions from trough to peak (t_1p_1 and t_2p_2, Fig. 1) remains fairly constant at 10-fold. It appears that during the 3 days of observation the IPF present in a serum dilution at any moment has been able to inhibit only one-tenth of the PF activated.

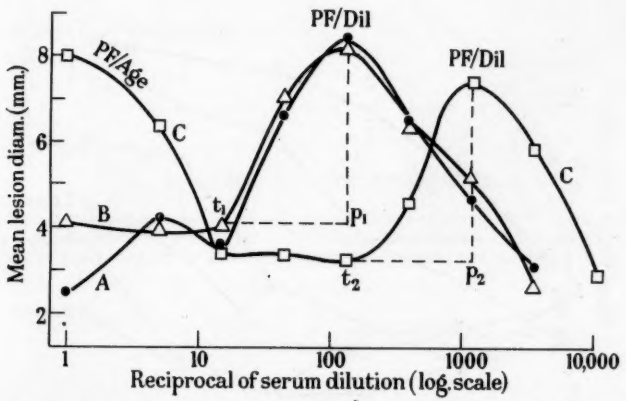

FIG. 1.—The activation of the permeability factor PF/Dil by dilution in 0·85 per cent saline of fresh serum from defibrinated blood (A) and from clotted blood (B), for 1½ hr. at room temperature, and from defibrinated blood for 3 days at 4° (C).

We stated previously (Miles and Wilhelm, 1955) that IPF might be activated to some extent by low dilution of serum, but that in general it appeared to be present in native serum in an active form. If there is no activation of IPF in high dilutions, the trough might move to the right as the result of a slow but progressive action of this existing IPF on PF/Dil. That this is so is evident from tests with G2 and G1S/P, the fractions rich in PF and IPF respectively. The same batch of G1S/P was used throughout these tests. The inhibiting potency of IPF in the fraction G1S/P was previously reported as maintained for 20 weeks at −10°. The material is still fully potent after 80 weeks at −10°. The G1S/P fraction can also be preserved in the freeze-dried state. It loses about 10 per cent of its potency in the process of freeze-drying.

A fixed dose of IPF (0·17 per cent) was mixed with graded concentrations (2–486 μg./ml.) of PF. The mixtures, and control solutions of G2 alone, were held for 45 min. at room temperature and then at 4°. Samples were assayed in

batches of 4 guinea-pigs after the preliminary 45 min., and after a further 1, 2, 4 and 9 days at 4°. The results of the five assays are shown in Fig. 2. The five curves for the control PF are combined into one. The inhibition of the highest concentration of PF is almost complete in 9 days, and a comparison of the curves between the 5 and 8 mm. response-levels gives the following approximate potency ratios between control and test mixtures, and the corresponding percentage inhibition :

Age of mixture	Potency ratio of PF to (PF + IPF)	Percentage inhibition of PF
45 min.	8	87
1 day	51	98
2 days	87	99
4 „	250	>99
9 „	425	>99

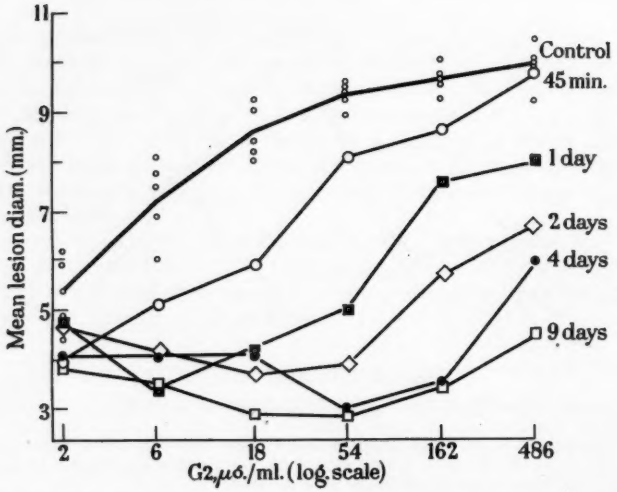

FIG. 2.—The progressive inhibition of PF/G2 by the IPF fraction G1S/P, 0·17 per cent, after 45 min. at room temperature and a further 9 days at 4°. (For μδ on abscissa read μg.)

The diameters of the lesions produced by the PF-IPF mixtures decrease by roughly equal amounts at 1, 2, 4 and 9 days, suggesting a linear decrease of log. potency with log. time. The same time-relation held for the reaction at 4° of three other concentrations of IPF. Fig. 3 records the decrease of log. potency with log. time for all four tests. Within 45 min. at room temperature, the curves differ considerably ; indeed, no inhibition is detectable with 0·006 and 0·018 per cent IPF ; inhibition is 50 and 87 per cent with the higher concentrations. But after 1–2 days at 4°, the curves are approximately linear.

It is clear, therefore, that the shift in the trough of inhibition in the dilution curves can be explained by slow action of pre-existing IPF, even in the low concentration existing in high serum dilutions, and there is no need to postulate the late activation of more IPF in higher dilutions.

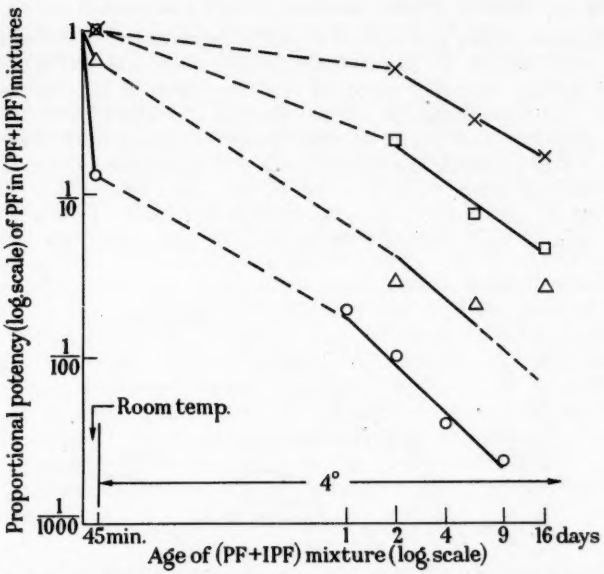

FIG. 3.—The progressive inhibition of PF/G2 by the IPF fraction G18/P. Each point represents the potency ratio of PF and a (PF + IPF) mixture, estimated from assays like that in Fig. 2. All mixtures were held for 45 min. at room temperature and thereafter at 4°.

IPF, 0·006 per cent, ×——×.
„ 0·018 „ „ , □——□.
„ 0·055 „ „ , △——△.
„ 0·165 „ „ , ○——○.

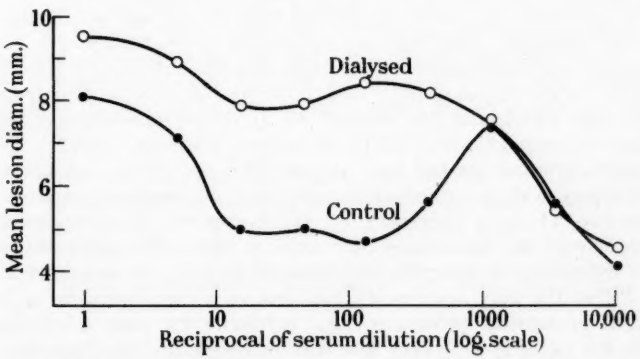

FIG. 4.—The effect of dialysis against 0·85 per cent saline for 3 days at 4° on the PF activity of serial dilutions of guinea-pig serum.

Modification of the dilution effect by dialysis

In our first paper (Miles and Wilhelm, 1955), we reported that dialysis of serum dilutions for one day at 4° did not materially alter the dilution curve. We restricted our tests to one day to avoid the formation of PF/Age, which might have confused the interpretation of our results. However, our colleague, Dr. J. M. Elder, subsequently observed that 3 days' dialysis at 4° against a twice daily change of saline altered the dilution curve of human serum (to be published) and that guinea-pig serum was affected in the same way. In control serum dilutions held at 4°, as already reported, PF/Age appears ; there is a wide trough of inhibition because the peak of PF/Dil activity has shifted to 1/1200 and the

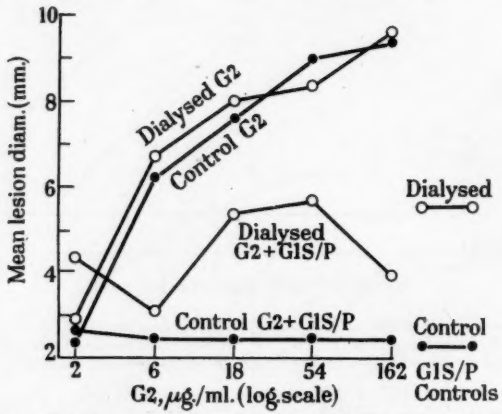

FIG. 5.—The failure of dialysis to restore the PF activity of G2 after its inhibition by the IPF fraction, G1S/P.

end-point to 1/10,000. In the serum dilutions dialysed for 3 days, the end-point of PF/Dil is also 1/10,000, but the trough of inhibition is almost completely abolished (Fig. 4). The result is a broad peak of activity, maximum at 1/200, as compared with the maximum at 1/2000 reached by serum dilutions allowed to age without dialysis. The activity appearing in the range 1/5–1/405 is not due to an exaggerated formation of PF/Age, but presumably to PF/Dil, because it is susceptible to the action of both G1S/P (IPF) and soya bean trypsin inhibitor (SBTI) ; whereas PF/Age is not affected by IPF and only slightly inhibited by relatively high concentrations of SBTI (Miles and Wilhelm, 1955).

Our previous work indicated that neither IPF nor PF is capable of diffusion through cellophan. It is nevertheless possible that dialysis removes some diffusible substance which is necessary for the continued combination of PF and IPF. Alternatively the substance may hold in check the activation of the PF precursor in the more concentrated solutions of serum ; for example, the conversion of pro-PF to PF may involve the removal of a small peptide, as happens in the activation of several proteases, *e.g.*, trypsin. In such a system, dialysis would dilute the peptide to a very low concentration and the reaction pro-PF → PF + peptide would be displaced far to the right. Substantial amounts of pro-PF in excess of the amount inhibitable by the IPF present would then appear

even in low serum dilutions. Finally, the dialysable substance might be a small-molecular inhibitor of PF activity, formed when PF and IPF combine; thus, PF as a protease may break down IPF to form polypeptides, which then inhibit the enzyme.

If this last explanation is true, it should be possible to remove the small-molecular inhibitor by prolonged dialysis of PF-IPF mixtures. Accordingly, final concentrations of G2, 2–162 μg./ml., were mixed with G1S/P in a final concentration of 0·17 per cent, and held for 45 min. at room temperature and a further 6 days at 4°; after this period, the inhibition in all mixtures was >99·9 per cent. The mixtures, together with control solutions of G2 and G1S/P, were then dialysed against twice-daily changes of a large volume of saline for 3 days at 4°, then for 3 hr. at room temperature, and immediately titrated in the skin. As Fig. 5 shows, the undialysed PF-IPF mixtures had no activity, and dialysed G2 was as active as undialysed G2. The dialysed PF-IPF mixtures showed some activity, but even in the most active mixtures, originally containing G2, 54 μg./ml., the intensity of blueing was very feeble (+ as against an expected + + +) and moreover, although G1S/P itself had no PF activity, dialysed G1S/P itself produced lesions of the same diameter and intensity as the dialysed G2-G1S/P mixtures. There is, therefore, no good evidence that the inactivity of PF-IPF mixtures is due to a diffusible inhibitor. The other possibilities are under investigation.

Improved Isolation of PF

The most potent PF obtained by applying the aqueous ethyl-ether method of Kekwick and Mackay (1954) to the fractionation of guinea-pig serum, was the fraction G2. The average PF potency of G2 can be increased 3-fold by the electrophoretic separation of the α- and β-globulin fractions, G2α having 18 times the potency of G2β. Preparative electrophoresis is not easy for bulk purification of PF, and we were able to produce fractions almost as potent as G2α by a modification of the precipitation technique. G2 is produced from G1 by bringing it to pH 5 and diluting with water to an ionic strength of 0·01. At this stage a precipitate forms; more precipitation occurs with the addition of 10 vol. per cent of ethyl-ether (Mackay, unpublished) and the combined precipitate is harvested as G2. By harvesting the first and second precipitates as G2/1 and G2/2, we achieved a separation of PF such that G2/1 had 8 times the potency of G2/2; and when G2/1 was re-precipitated under the same conditions, the resulting G2/1R had up to 18 times the potency of G2/2. The potencies of these various preparations in terms of histamine are set out in Table I, together with the results of electrophoretic analysis.

TABLE I.—*Electrophoretic Protein Components, and PF Potency Relative to Histamine, of various G2 Fractions (see text)*

Expt.	Batch	Fraction	Percentage protein content by electrophoresis				PF potency relative to histamine
			Albumins	Globulins			
				α	β	γ	
1	A	G2	—	49	50	—	0·36
2	B	G2α	—	>90	<10	—	1·14
3	33	G2/1R	—	50	49	—	0·6
4	33	G2/2	—	42	48	9	0·03

A is the mean of 6 batches, B of 7 batches.

G2/1R is almost as potent as G2α, but the content of α-globulin differs widely. Clearly the PF is not the only α-globulin in these preparations. The improvement in potency during the step from G2/1 to G2/1R is not only a removal of inert material, because G2/1R, though twice as potent, contains 80–90 per cent of the protein in G2/1. The increase in potency is due either to the activation of more PF, or, what is more likely in view of the fact that IPF is an α_1-globulin, the removal of traces of inhibitor.

The effect of an antihistamine drug on PF/G2

We reported that the general blueing of the skin after the intravenous injection of PF/G2 into guinea-pigs with circulating dye, and the local blueing on intracutaneous injection of PF/G2, were not affected by mepyramine maleate, 20 mg./kg. given intraperitoneally 2 hr. before the test. The conclusion that PF/G2 is not a histamine-liberator is based on the assumption that an effective systemic dose of the mepyramine was used.

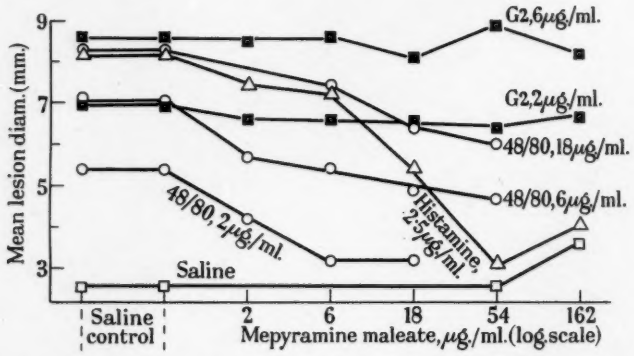

FIG. 6.—The insusceptibility of PF/G2 to local mepyramine maleate, injected intracutaneously with the PF.

The experiments were repeated, using mepyramine as a local inhibitor, mixed with approximately equipotent doses of histamine, 48/80 and PF/G2 before intracutaneous injection, in the hope of producing local concentrations of mepyramine in excess of the highest local concentration obtained after intraperitoneal injection. Intracutaneous mepyramine, however, in concentrations above 162 μg./ml. itself increases permeability. Its action on the three permeability factors was tested in final concentrations up to this level. The mixtures were held for 30 min. at room temperature before injection. The tests were repeated more than once, with substantially similar results. Fig. 6 summarizes the results of two tests in which the similarity of the animals on the two occasions was established by an identical response to histamine. The effect of histamine, 2·5 μg./ml. was abolished by 54 μg./ml. of mepyramine. In this concentration, mepyramine decreases the mean diameter of the lesions produced by 2, 6 and 18 μg./ml. of 48/80 to approximately the same extent, 2·2 mm. Since the average slope of the 48/80 dosage-response is 2·5, this is equivalent to an antilog. (2·2/2·5) = 7·5-fold decrease in potency. Two, 6 and 18 μg./ml. of mepyramine are proportionally

as effective on the high concentration of 48/80 as they are on the low. In contrast, G2/1R at both 2 and 6 μg./ml. is unaffected by mepyramine, 2–162 μg./ml.

Histological effects of intravenous PF/G2

Four guinea-pigs were shocked acutely (Wilhelm *et al.*, 1955) with 10–20 mg./kg. of PF/G2 intravenously. The following tissues were sampled 48 hr. later, and examined histologically: trachea, lung, heart, tongue, salivary gland, stomach, ileum, transverse colon, liver, spleen, pancreas, thyroid, thymus, adrenal, kidney, bladder, testis, ovary, diaphragm, muscle from the trunk and limbs, skin, bone-marrow and brain.

The PF/G2 had induced no microscopic changes in any of the tissues. The livers of three animals, like those of untreated controls, contained small tuberculoid lesions. There are indications that G2 is a protease; but there were no signs in cardiac and skeletal muscle of the focal necrosis which proteolytic enzymes induce in rabbits, rats and mice (Kellner and Robertson, 1954).

Stability of the Serum PF-IPF System In Vivo

We do not yet know whether PF, or the precursor from which it appears to be so readily formed, or IPF exists in the body outside the blood. It is however reasonable at this stage to assume at least that tissues other than blood play some part in the synthesis and destruction of these substances, and that the PF-IPF system in serum would be dependent on the integrity of some general bodily functions. In view of our belief that PF and IPF are involved in inflammation and perhaps other pathological phenomena, we investigated the sera of guinea-pigs subjected to bacterial infection, irradiation and various kinds of shock. Since the dilution effect depends on the relative concentration of IPF and of PF-precursor in the blood, and on the rate of activation of PF and its inactivation by IPF, we used it as an index of normality of the PF-IPF system.

The main features of the "normal" dilution effect are shown in Fig. 1. Any gross change in the relative quantities, potencies, activation rates or combination rates of PF and IPF, might affect the normal dilution curve; thus, the inhibition trough exemplified in Fig. 1 might be diminished by excess activation of PF, or by a destruction of IPF; and the PF peak might decrease because the precursor of PF or substances necessary for its activation were lacking.

Bacteraemic infection

Guinea-pigs were infected by the intravenous injection of a group C streptococcus (Wellcome Collection, CN 771). The dose of about 10^6 viable cocci produced a febrile and wasting disease accompanied by continuous bacteraemia, some 80 per cent of the animals dying within 16 days. Animals surviving the first 7–10 days developed either joint abscesses or haemorrhagic pericarditis, and sometimes both.

On the 2nd, 4th, 8th, 12th and 16th days of the disease blood was obtained by cardiac puncture from 3 infected animals and 1 control, immediately defibrinated, and centrifuged to remove cells; the resulting sera were held at 4° until diluted. The serum dilutions were prepared within 2 hr., held at room temperature for 1¼–1½ hr., and tested in normal guinea-pigs. The position of

the trough and peak were normal, and the only abnormality was the appearance of a PF in low dilutions, corresponding to PF/Age (Miles and Wilhelm, 1955) in about half the specimens tested; see Fig. 7.

X-irradiation

Guinea-pigs of our laboratory strain, fed on water and the pelleted diet no. 18 of Bruce and Parkes (1947) with a supplement of cabbage and hay, were subjected in pairs to doses of 100, 150, 200, 300 and 400 r. The dosage rate was 29 r/min. whole body irradiation (250 kvp, 15 mA., 1·5 mm. Cu H.V.L.) and the dosage was graded by length of exposure. The most suitable dose for a non-lethal bout of irradiation sickness was 150 r, the effect of which on haemoglobin, leucocyte count and weight is typified in Fig. 8. Accordingly, 30 guinea-pigs were subjected to this dose and observed for 29 days. On the 6th, 12th, 18th

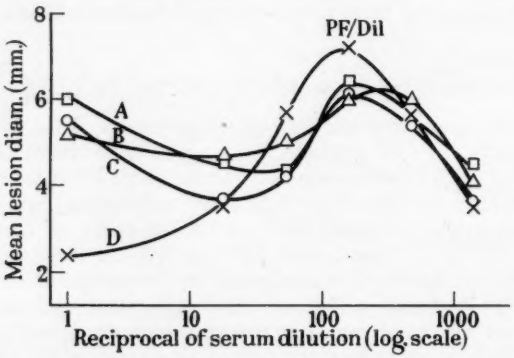

FIG. 7.—The activation of PF/Dil in the sera of three guinea-pigs (A, B and C) after 8 days' bacteraemic infection by a group C streptococcus. D = normal serum.

and 27th days, a period covering the onset, duration of and recovery from irradiation sickness, blood samples were taken from 2 irradiated animals and 1 normal. After activation, each group of serum dilutions was tested in 4 normal animals and, except on the 18th day, when all the available irradiated animals were too liable to skin haemorrhages on handling to be tested, in 4 irradiated animals. All the 30 irradiated animals, as judged clinically and by 3-daily estimates of weight and blood haemoglobin, underwent a typical attack of irradiation sickness; in 4 of the 30 it was fatal.

Sera from irradiated guinea-pigs had the same reaction pattern as normal sera, when tested either in normal or irradiated guinea-pigs.

Cortisone

The sera of 3 guinea-pigs that had received respectively 3, 6 and 9 daily doses of 5 mg. cortisone acetate/kg. body weight subcutaneously were similar to normal sera as regards the PF/Dil peak, but in the undiluted sera of the two most heavily dosed animals, the PF corresponding to PF/Age was more active than in untreated controls.

It is to be noted in this connection that the 3–9 days' treatment did not diminish the susceptibility of guinea-pigs to the serum permeability factors, or to histamine, 48/80 and leukotaxine. In fact, all lesions produced by these substances were very slightly larger than in the controls, a result that may have been due to the higher blood pressure and consequently higher exudation pressures in the treated animals. The average pressure in the main central artery of the ear, measured by a modified Grant's capsule (Miles and Niven, 1950), was 30 mm. Hg in the treated animals and 10 mm. Hg lower in the controls.

Shock

Active anaphylaxis.—Five guinea-pigs sensitized 15–20 days beforehand by 0·1 ml. of fresh rabbit serum subcutaneously, were subjected to acute anaphylactic shock by the intravenous injection of 0·25 to 0·5 ml. of rabbit serum. After $1\frac{1}{2}$–$7\frac{1}{2}$

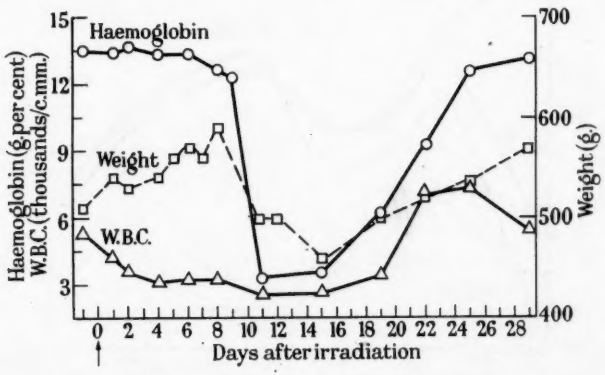

FIG. 8.—The typical changes in haemoglobin concentration, total white blood cell count (W.B.C.) and weight of the test strain of guinea-pig, for 28 days after X-irradiation (150 r).

min. blood was obtained by stunning the animal and cutting its throat. In some instances the serum was obtained from a clot, in others by spinning the defibrinated blood. The blood in all cases was slow to clot, the fibrin yield appeared to be small and the serum was discoloured as a result of haemolysis. The proportional yield of serum was low.

In none of the five sera was there any abnormality of the PF/Dil peak ; but, as Fig. 9 shows, lesion diameters in the trough of inhibition (1/5 to 1/45) were greater than usual, and in some specimens the PF activity in undiluted serum was high. Moreover, the secondary peak at 1/5 found in many specimens of " defibrinated " serum was exaggerated. The effect was not due to the rabbit serum used as the intravenous shocking dose, because in an unsensitized animal a similar dose did not modify the dilution pattern of its serum.

Passive anaphylaxis.—Mild anaphylactic shock was induced in 3 guinea-pigs 2 days after sensitization with intraperitoneal anti-pneumococcal SIII antibody, 0·2 mg. antibody N/kg., by the intravenous injection of 5 times the serologically equivalent dose of SIII polysaccharide. The blood was sampled 6, 6 and 45 min.

after the shocking dose, and defibrinated. The dilution pattern of the sera was normal.

By compound 48/80.—Moderate to acute anaphylactoid shock was induced in 3 guinea-pigs by the intravenous injection of the histamine-liberator, compound 48/80 (Paton, 1951), 1·0–1·5 mg./kg. The reactions to dilutions of the sera from blood collected 4, 7 and 9 min. later were normal.

By dextran sulphate.—Anaphylactoid shock induced by the intravenous injection of large-molecular dextran sulphate (Walton and Ricketts, 1954) was the only pathological modification attempted which altered the dilution pattern of serum. Four preparations of dextran sulphate were tested. They were supplied by Drs. Walton and Ricketts, who determined the properties summarized in the first five entries of Table II.

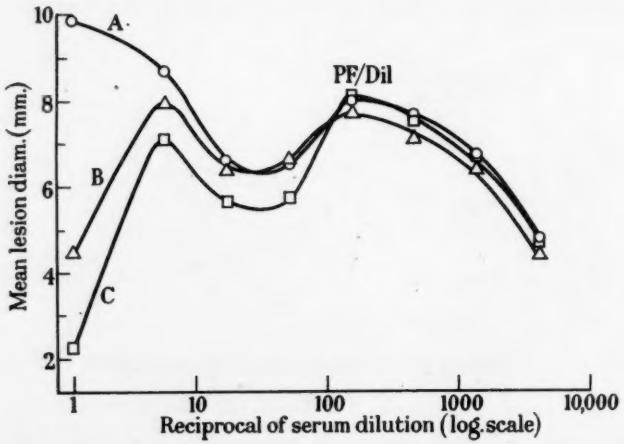

FIG. 9.—The activation of PF/Dil in the sera from three guinea-pigs (A, B and C) in acute anaphylactic shock. The activity of the PF in low dilutions, corresponding to PF/Age, is increased to varying degree.

TABLE II.—*Properties of Various Dextran Sulphates Tested for their Effect on the Activation of PF/Dil*

Property	Serial no. of dextran sulphate			
	GA	D/2	E/2	I/7/5
Molecular weight	1,000,000	515,000	150,000	<7,000
Intrinsic viscosity of parent dextran	0·62	0·36	0·12	0·02
Sulphur content (per cent)	16·1	17·8	12·6	19·6
Anticoagulant activity (heparin units/mg.)	72	68	20	13
Induction of anaphylactoid reaction in guinea-pigs	+++	+++	+	0
Effective blueing dose (μg. in 0·1 ml.)	2	2	4	125
Maximum non-blueing dose (μg. in 0·1 ml.)	0·3	0·3	0·6	32

ERRATUM

Vol. XXXVIII, No. 4, August 1957 (D. L. Wilhelm, P. J. Mill and A. A. Miles.)

The graph entitled **Fig. 10, p. 457,** should appear on p. 458 above the description given there for Fig. 11, and *vice versa*.

Guinea-pigs were given 2·5 mg./kg. of dextran sulphate, D/2, intravenously. The animals became shocked after ½ min. and were still in shock when killed after 30–45 min. Blood samples for defibrination were taken 1½–4 min. after the injection. The dilution pattern of the serum was modified by a partial or complete suppression of the peak of activation around the 1/135 dilution (Fig. 10). The shock induced by 0·5 mg./kg. of D/2 was less severe and the dilution pattern of the sera from two such shocked animals was normal.

The modification of the serum, however, is clearly not secondary to dextran sulphate intoxication of the animal because the *in vitro* treatment of fresh normal guinea-pig serum with dextran sulphate, in concentrations of the same order as those obtaining in the blood of animals given the drug intravenously, suppressed the dilution effect to the same extent. Thus, assuming a plasma volume of

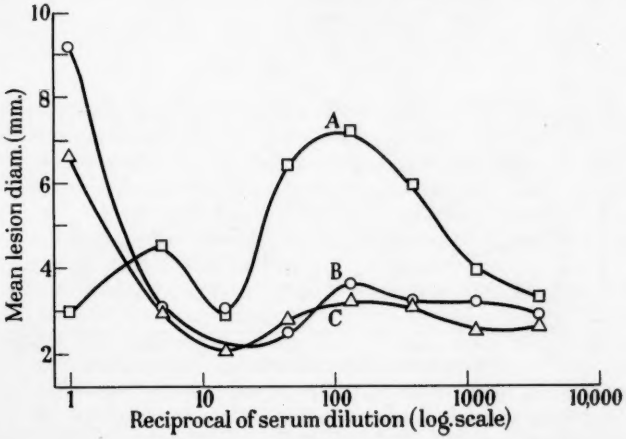

FIG. 10.—The partial (B) or complete (C) suppression of the activation of PF/Dil in serum from guinea-pigs shocked with intravenous dextran sulphate, D/2, 2·5 mg./kg. PF/Dil was normally activated in serum (A) from animals given 0·5 mg./kg.

40 ml./kg. (Ancill, 1956), in animals receiving a shocking dose of 2·5 mg./kg. of D/2, the serum concentration will be about $\frac{2{\cdot}5 \times 10^3}{40} = 60$ μg./ml. Fresh serum held at room temperature with D/2 in final concentrations of 33 and 100 μg./ml. for 45 min. before dilution, had a dilution pattern (Fig. 11) which was almost identical with that of sera from animals in dextran sulphate shock. The same effect was produced by 15 min. and 2 hr. pre-treatment with the D/2.

Dextran sulphate itself is a permeability factor, preparations of higher molecular weight being the more potent. The last two rows of Table II record the effective blueing doses (EBD) and the maximum non-blueing doses of the four preparations in guinea-pig skin (the EBD is the amount in 0·1 ml. producing a 6 mm. lesion in the blued animal). Preparation I/7/5, with a molecular weight of <7000, is feebly potent. The remainder are about equally potent, but none increases permeability in the skin test in concentrations of <3 μg./ml. Since the

PF/Dil peak is suppressed in a dilution range of 1/50–1/1000, in sera treated with 100 μg./ml. (Fig. 11, B), the final concentration of D/2 in this range is 0·1–1·0 μg./ml. which is well below the maximum non-blueing concentration. The moderate activity at 1/100 exemplified in Fig. 10, B, in which the estimated concentration of D/2 is 0·6 μg./ml., is due therefore to some activated PF/Dil and not to the low concentration of dextran sulphate present. The relatively high activity of undiluted serum in the curves B and C in Fig. 10 and 11 is of the order expected from the contained D/2.

The efficacy of the four preparations in suppressing activation of serum was tested in sera pre-treated for 45 min. at room temperature with each preparation, in concentrations of 33, 100 and 300 μg./ml. PF/Dil was activated by diluting the sera 1/405 and holding the dilutions for 75 min. at room temperature. In the dilution pattern of normal serum, the 1/405 dilution, being beyond the plateau

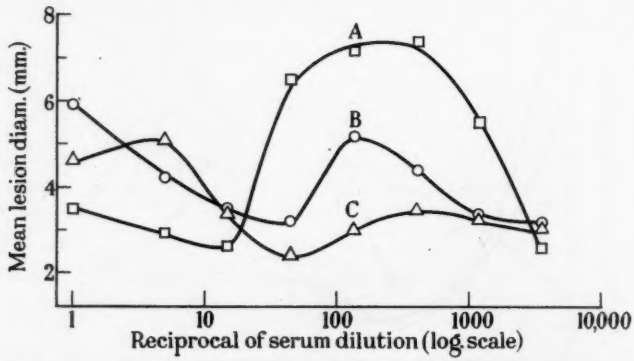

FIG. 11.—The suppression of the activation of PF/Dil in serum pre-treated *in vitro* with dextran sulphate, D/2, in final concentrations of 33 μg./ml. (C) and 100 μg./ml. (B). A = saline-treated control.

of peak activity (*cf.* Fig. 11, A), is clearly a sensitive system for detecting changes in activation. The approximate potency of the PF/Dil in this dilution was calculated from the difference (d) in mean lesion-diameter between control and treated sera. Since the average declining slope of PF/Dil titrations of lesion-diameter against log. concentration is about $-3{\cdot}5$, the factor of increase of potency is antilog. (d/3·5). These estimated values are listed in Table III, the controls being cited as unity. The preparation I/7/5, mol. wt. <7000, did not substantially affect the activation of PF/Dil and needs no further consideration. Of the other preparations, at a concentration of 33 μg./ml. only GA suppressed PF/Dil; at 100 μg./ml. all decreased the PF potency 3- to 4-fold; at 300 μg./ml., all *increased* the potency. Hence there is an optimum concentration of dextran sulphate for the suppression of activation. The increase in PF potency by pre-treatment with concentrations of 300 μg./ml. is not due to an intrinsic activity of the dextran sulphates, because at 1/405 their concentration is 0·75 μg./ml. It may be due to an activation by the high concentration, or a synergic action of the dextran sulphates and PF/Dil. Serum was pretreated

with dextrans similar to the parent dextrans from which the sulphates GA, D/2, E/2, and I/7/5 were prepared. The pre-treatment with each dextran in final concentrations of 33, 100 and 300 μg./ml. did not affect the activation of PF/Dil.

TABLE III.—*PF/Dil Potency, Relative to a Control Preparation, of* 1/405 *Dilutions of Sera Treated before Dilution with* 33, 100 *and* 300 μg./ml. *of Dextran Sulphates of Various Molecular Weights*

Dextran sulphate		Concentration of dextran sulphate (μg./ml.) in pre-treated serum		
Serial no.	Mol. wt.	33	100	300
GA	1,000,000	0·27	0·23	1·2
D/2	515,000	1·0	0·25	1·2
E/2	150,000	1·0	0·32	2·5
I/7/5	<7,000	1·0	1·2	1·2
Control		1·0	1·0	1·0

A similar increase in potency over the whole peak of PF/Dil activation occurred when D/2, in final concentrations of 1 and 3 μg./ml., was allowed to act for 45 min. at room temperature on the full series of dilutions already matured for 90 min. on the bench. Similarly, when preparation GA, in final concentrations of 0·027–2·22 μg./ml. was allowed to act for 45 min. at room temperature on portions of a matured 1/405 dilution, the mixtures containing GA had 2–4 times the PF potency of a portion mixed with saline.

It is clear that dextran sulphate, with a relatively high sulphur content and a high molecular weight, in an optimal concentration depending on the preparation used, will inhibit the activation of PF/Dil; but it does not inhibit the intracutaneous permeability effect of already activated PF/Dil. The relevance of this effect to dextran sulphate shock, and to the mechanism of the dilution effect, is under investigation. It may be noted that a similar modification of the dilution effect is produced in human serum by the related anticoagulant leparin (J. M. Elder, unpublished).

Taking these largely negative results at their face value, we may guess that if the PF-IPF system, as manifested in blood, plays any part in natural or pathological phenomena, any alterations in its normally inactive state are induced locally either in affected vessels or in the tissue spaces themselves. It is probable that substantial alterations of the system in the blood itself will be induced only by procedures that materially affect either the synthesis or the normal degradation of the PF and IPF proteins.

In summary, the deviations from the normal pattern of the dilution curves exhibited by these "pathological" sera were not sufficient to warrant their description as abnormal, although in general a greater proportion of them than is usual had relatively high PF activity in the lowest serum dilutions. This was particularly evident in serum after active anaphylaxis. We conclude that the main PF-IPF system in the blood is relatively stable. It must nevertheless be emphasized that the dilution curve, which is the result of the interaction of many factors, may be an insensitive indicator of change. When it becomes possible to assay the factors separately, significant serum abnormalities might be revealed in these and other pathological conditions.

SUMMARY AND CONCLUSIONS

Our further observations on the permeability factor and its inhibitor of *normal* guinea-pig serum, mainly refinements on previously reported work, are as follows :

1. The main permeability factor (PF) of guinea-pig serum is activated maximally in one hour at room temperature by dilution in saline to 1/200. Little PF activity appears in lower dilutions one hour old owing to the action of IPF, the serum inhibitor of PF. This trough of inhibition, in dilutions of 1/2–1/30, is removed by dialysis against saline for 3 days at 4°. The substance affected by dialysis does not appear to be a small-molecular inhibitor resulting from an interaction of PF and IPF.

2. The PF activity of serum one hour after dilution at 1/200 disappears when the serum dilution is held for 3 days at 4°, owing to combination of PF/Dil with the low-potency native IPF present. In the reaction of isolated preparations of PF and IPF, although 80 per cent of PF is inhibited within the first hour, the reaction is slowly progressive and 0·25 per cent of the original activity remains after 9 days.

3. The aqueous ethyl-ether technique of fractionating serum can be modified so as to isolate the PF with a potency previously attained only by electrophoretic separation.

4. The permeability effect of PF is insusceptible to a local concentration of 0·16 mg./ml. mepyramine maleate.

5. The intravenous injection of optimal hypotensive shocking doses of the PF does not induce any histological change in the principal organs detectable after 48 hours.

Our attempts to demonstrate changes in serum PF and IPF in a few gross *pathological* conditions were with one exception negative.

6. The PF-IPF system in the serum of the guinea-pig appears to be relatively stable. As judged by the normality of the dilution effect, it is not substantially modified in guinea-pigs subjected to

(*a*) Acute streptococcal infection.
(*b*) X-irradiation sickness.
(*c*) A 9-day course of cortisone treatment.
(*d*) Acute anaphylaxis, active and passive.
(*e*) Shock induced by the histamine-liberator, compound 48/80.

Dextran sulphates, with a sulphur content of 12–18 per cent and a molecular weight of 150,000–1,000,000, in optimal concentrations depending on the preparation used, inhibit the *in vitro* activation of PF/Dil, whether added to blood by intravenous injection in the living animal, or to fresh serum *in vitro*.

We are indebted to Miss E. B. Harriss, Institute of Cancer Research, the Royal Cancer Hospital, London, for irradiating guinea-pigs ; to Dr. J. H. Humphrey, National Institute for Medical Research, Mill Hill, London, for the pneumococcal SIII polysaccharide and its antibody ; to Dr. E. J. de Beer, Wellcome Research Laboratories, Tuckahoe, U.S.A., for compound 48/80 ; to Dr. K. W. Walton, Department of Experimental Pathology, University of Birmingham, and Dr. C. R. Ricketts, Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham, for preparations of dextran and dextran sulphates ; to our

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THE PERMEABILITY-INCREASING FACTOR IN DILUTED HUMAN PLASMA

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THE mechanisms controlling normal capillary permeability, and those increasing permeability in many pathological states, are largely unknown. Recently, Mackay, Miles, Schachter and Wilhelm (1953) have demonstrated the activation of potent permeability-increasing factors in guinea-pig serum. In an extension of this work, Miles and Wilhelm (1955) have described the conditions under which simple dilution of guinea-pig serum activates a factor (PF/Dil) and under which storage of guinea-pig serum activates a factor (PF/Age). Wilhelm, Miles and Mackay (1955) have characterized PF/Dil as an enzyme-like α-globulin. These and other authors (Editorial, 1955, 1956) have commented on the possible rôle of PF/Dil in inflammation, but the possibility has not been mentioned that PF/Dil may be of equal importance in the control of normal capillary permeability or in the increase of permeability in those oedema states where no inflammation is present.

During some experiments on plasma injected into the pleural cavity of dogs, we became interested in permeability-altering factors in plasma, for it was clear that some of the results were due to altered pleural capillary permeability. We have examined the plasma of several species for the presence of PF/Dil and PF/Age, and have found that a substance resembling PF/Dil is very easily demonstrated in human subjects. This finding, and the possibility that this substance may be significant *in vivo*, have led us to do a series of experiments to determine the simpler characteristics of this substance in man.

METHODS

The skin was used as the test site. Before testing, each subject received an intravenous injection of T1824 (Evans blue), a dye which binds securely to the plasma albumin (Rawson, 1943). When test fluids were thereafter injected intradermally, any local increase in capillary permeability was detected by the appearance at the injection site of blue staining due to the escape of dye from the circulation.

Healthy young adult males were used as subjects. It was found by experience that subjects who exhibited dermatographia were unsuitable, for they show marked triple responses to any intradermal injection. Subjects were therefore screened by the intradermal injection of 0·1 ml. of isotonic saline and rejected if a triple response was present after 30 min. Of 13 volunteers, 3 were rejected because of this phenomenon.

All apparatus was sterilized by dry heat. Five ml. of blood were obtained by venepuncture and heparinized with 0·1 ml. of 1 per cent sodium heparin in isotonic saline. The plasma was separated by centrifugation at 3000 r.p.m. for 10 min. The plasma was serially diluted with each of 3 diluents: (i) isotonic saline, (ii) isotonic saline containing 0·01 mg./ml. of the antihistaminic mepyramine maleate, and (iii) isotonic saline containing 0·1 mg./ml. of crystal-

line soya bean trypsin inhibitor (Worthington). The diluted samples were kept at room temperature for 1 hr. before testing.

The subject from whom the blood sample was obtained was given 50 mg. of T1824 (Warner Chilcott) in 10 ml. of saline intravenously. This dose produced marked staining of the plasma, but the skin colour remained normal so long as capillary permeability was unaltered.

Test sites were placed at random within a grid marked with ink on the back. A volume of 0·1 ml. of each diluted sample was injected intradermally with a 26 s.w.g. short-bevel needle. Control injections included the 3 diluents without added plasma, undiluted plasma, and 4 histamine solutions made up from a stock solution of 1 mg./ml. (calculated as the base) to a final concentration of 1 μg./ml. with each of the 3 diluents and with undiluted autologous plasma. The diameter of the blued area at each injection site was measured by an independent observer 30 min. after injection.

Samples of plasma from 3 subjects were stored at 4° under sterile conditions for periods up to one week, and then tested undiluted for evidence of any factor resembling PF/Age.

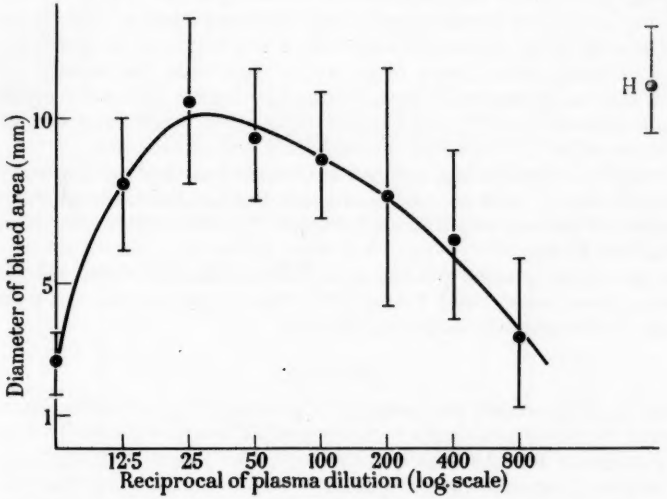

FIG. 1.—Responses to intradermal injection of autologous plasma diluted with saline. The value at each dilution represents the mean ± one S.D. of readings from 7 subjects (one reading per subject). Value at extreme right (● H) represents response to 0·1 μg. histamine.

RESULTS

In each of the 10 subjects tested, autologous plasma diluted with isotonic saline produced an increase in capillary permeability as judged by the appearance of blueing at the injection site. The results in 7 subjects, in whom a full range of dilutions was studied, are shown in Fig. 1. Blueing appeared rapidly, usually within 1–2 min. after injection. The largest responses were produced on the average by a 1/25 dilution, although considerable activity was still present at a 1/400 dilution. Undiluted plasma produced no more blueing than could be accounted for by the trauma of the needle puncture. It should be noted that without the blueing technique, the increase in capillary permeability produced by diluted plasma might easily be overlooked, for the wheal resulting from

increased permeability can readily be confused with the bleb raised by the volume of injected fluid.

Histamine, 0·1 μg. in 0·1 ml. of saline, produced a blued area with a diameter only slightly larger than that produced by a 1/25 dilution of plasma. However, a striking difference was noted between the responses to histamine and those to diluted plasma. Histamine in either saline or plasma produced blue wheals which were still surrounded by marked flares after 30 min. The wheals produced by diluted plasma, on the other hand, were in no instance surrounded by flares when read at the same time.

The addition of 0·01 mg./ml. of mepyramine maleate reduced by about half the mean diameter of the blued area produced by 0·1 μg. of histamine, and markedly reduced the intensity of the blueing. This concentration of mepyramine did not alter significantly the responses to diluted plasma (Fig. 2). A concentration of 0·1 mg./ml. of soya bean trypsin inhibitor completely abolished the response to diluted plasma (Fig. 3), but did not affect the response to histamine. When its effect was being tested, soya bean trypsin inhibitor was usually included in the diluent, and was therefore present during incubation, but a few supplementary experiments showed that it was equally effective in abolishing the response to diluted plasma when it was added immediately before injection.

In all subjects, the diluents without added plasma produced no response.

A few preliminary tests showed that there was no difference in response when serum instead of plasma was diluted, or when Tyrode's solution instead of saline was used as the diluent.

Stored undiluted plasma produced no response in the three subjects tested. The plasmas tested were 3 and 7 days old. Stored plasma was not tested for the appearance of permeability factor on dilution.

DISCUSSION

In order to demonstrate the presence of permeability factors in human plasma, it is essential to use the subject's own plasma (autologous plasma) for testing, for we have previously found (Bliss and Stewart, 1957) that undiluted plasma from another subject (non-autologous plasma) can produce an increase in capillary permeability when injected intradermally. This phenomenon is not encountered with undiluted autologous plasma.

The results of the present investigation indicate that simple dilution of human plasma activates a potent factor capable of increasing capillary permeability. The factor appears to be the same as PF/Dil, described by Mackay *et al.* (1953) and Miles and Wilhelm (1955) in guinea-pig serum. Both factors are activated by dilution, and increase capillary permeability. The effect on capillary permeability in both cases is abolished by soya bean trypsin inhibitor, but is unaffected by antihistaminics. The most significant difference between the characteristics of these factors is that, after one hour's incubation, human plasma or serum possesses its greatest activity in the dilution range 1/12·5–1/100, whereas guinea-pig serum possesses it in the dilution range 1/100–1/400.

Miles and Wilhelm (1955) provide good evidence that the effect of diluted guinea-pig serum is not mediated by histamine. This appears to be true of the effect of diluted human plasma as well. The response is unaffected by antihistaminics, and, because of the absence of flaring, it is strikingly different in appearance from the response to injected histamine.

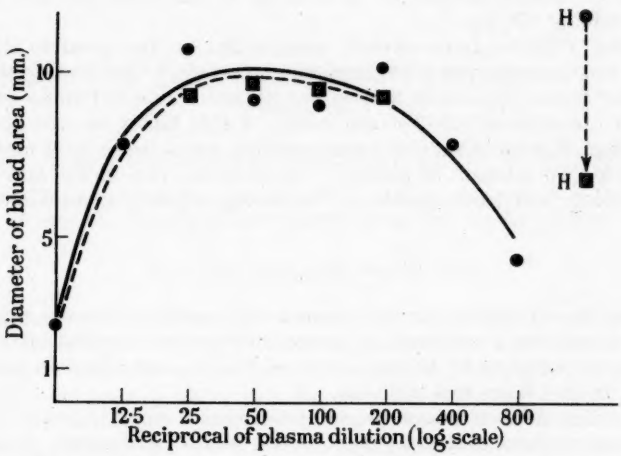

FIG. 2.—Effect of addition of an antihistaminic to diluent.

●———● isotonic saline as diluent, 5 subjects.
■- - - - -■ isotonic saline + 0·01 mg./ml. mepyramine maleate as diluent, same 5 subjects.

Values at extreme right represent responses to 0·1 μg. histamine in saline (● H) and in saline + 0·01 mg./ml. mepyramine maleate (■ H).

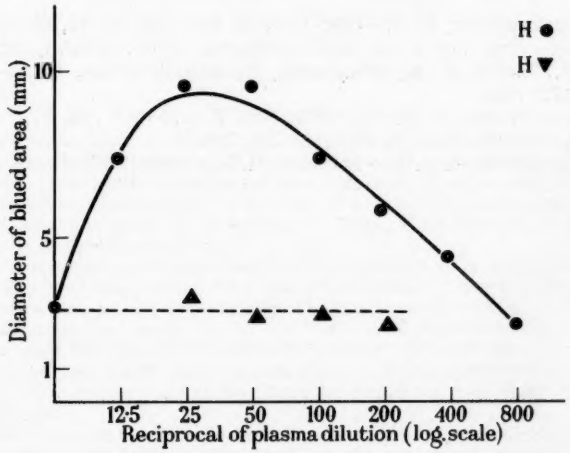

FIG. 3.—Effect of addition of soya bean trypsin inhibitor to diluent.

●———● isotonic saline as diluent, 3 subjects.
▲- - - - -▲ isotonic saline + 0·01 mg./ml. soya bean trypsin inhibitor as diluent, same 3 subjects.

Values at extreme right represent responses to 0·1 μg. histamine in saline (● H) and in saline + 0·01 mg./ml. soya bean trypsin inhibitor (▼ H).

Storage of human plasma for periods up to one week did not activate any factor resembling PF/Age.

Miles and Wilhelm have already commented on the possible significance of PF/Dil in the vascular phenomena of inflammation. Our finding that a similar permeability factor appears in a very low dilution range in human subjects raises the further question of whether activation of this factor *in vivo* plays a rôle in the regulation of normal capillary permeability, since tissue fluid may be thought of as a 1/10–1/20 dilution of plasma. In addition, this factor may play a rôle in perpetuating fluid accumulation by increasing capillary permeability in oedema states.

SUMMARY

Dilution of autologous human plasma with saline activates a potent factor capable of increasing local capillary permeability when injected intradermally.

This factor appears to be the same as PF/Dil, described in guinea-pigs by Mackay *et al.* and Miles and Wilhelm.

The fact that diluted human plasma possesses its greatest activity in relatively low dilutions suggests that activation of this factor *in vivo* may be significant in the control of normal capillary permeability and in various oedema states.

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THE ROUTE OF IMMUNIZATION IN TRANSPLANTATION IMMUNITY

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IT is commonly believed that all routes of administration of homologous tissue cells are equally effective for the elicitation of transplantation immunity. That this view needs at least some qualification was made clear by Medawar's (1946*a*) finding that suspensions of leucocytes are very much more effective in inducing immunity in the rabbit when injected intradermally rather than intravenously. This peculiarity of the intravenous route in rabbits also extends to suspensions of epidermal cells, for it has been reported by Billingham and Sparrow (1955) that a dosage of epidermal cells sufficient to elicit a powerful immunity if injected intradermally, fails to elicit any detectable immune response when injected intravenously. Indeed, a significant *prolongation* of the survival time of test skin homografts obtained from the cell donor was usually the outcome.

The subject matter of this paper is a comparative study of the immunological consequences of injecting homologous cells by various routes in the three most common laboratory mammals : mice, rabbits and guinea-pigs.

MATERIALS AND METHODS

Principle of the Experiments

A state of transplantation immunity, or heightened resistance to homografts, cannot at present be identified satisfactorily by any *in vitro* test. It must therefore be identified by following the fate of test grafts transplanted to a putatively immune subject from the donor whose cells it had previously received, or from another donor of similar genetic constitution. The most convenient test grafts are furnished by skin, or by portions of a transplantable tumour indigenous to the donor strain.

When a skin homograft is transplanted to an immune host it undergoes a more rapid destruction than when transplanted to a normal one, and it never enters that transient proliferative phase which is characteristic of grafts on untreated animals. A state of immunity may therefore be diagnosed according to the histological appearance of a test skin graft biopsied 6 days after its transplantation and confirmed, if necessary, by determining the survival time of the test grafts from examination of biopsy specimens taken at regularly spaced intervals. The survival times may then be compared with those relating to grafts on untreated animals.

In the tests with tumour homografts a small piece of viable tumour tissue having the same genetic origin as the injected cells is implanted into the host whose immunity is in question. Then, after a suitable period—which must, of course, be significantly shorter than the *median survival time* (M.S.T.) of tumour homografts in normal hosts—the graft is removed and its viability determined by implanting fragments of it into mice of its own strain of origin.

In all the experiments to be described the recipients were challenged with their test grafts 8–10 days after they had been injected with homologous cells by one route or another.

* Research Fellow of the British Empire Cancer Campaign.

Experimental Animals and Control Data

Mice

For the tests in which skin homografts were employed, adult mice belonging to the CBA and A strains were used as donors and recipients respectively. With this combination the M.S.T. of skin homografts transplanted to normal untreated animals is known to be 10·2 ± 0·3 days, and it is also known that graft destruction does not commence until after the 6th post-operative day (Billingham, Brent, Medawar and Sparrow, 1954). Evidence of any destruction in progress in a 6-day biopsy specimen therefore indicates immunity.

For the experiments in which tumour tissue test grafts were used, suspensions of thymocytes were used as the antigenic stimulus. Donors and recipients belonged respectively to the C57 BR/a and A strains. Sarcoma S778, which originated in, and takes uniformly in, C57 BR/a mice was used to challenge the recipients, fragments being implanted subcutaneously by trocar. The biological test for survival of the tumour implants was carried out after they had lain for 8 days in the A-strain hosts. As Table I*b* shows for the controls, 13/14 tumour implants removed after 8 days from normal A-strain mice gave evidence of viability, whereas none of the tumour test grafts removed from 12 animals which had previously reacted against and destroyed homografts of the tumour gave evidence of survival.

Rabbits

Because inbred strains were unavailable, donors and recipients were deliberately selected from different breeds to obtain the maximal degree of genetic disparity—another method of obtaining uniformity of response from different recipients to skin homografts from different donors (Billingham, Krohn and Medawar, 1951). Each rabbit received 3 small pinch grafts of its donor's skin, beds of the appropriate size being prepared in the side of the lateral thoracic wall. These grafts provided biopsy specimens for removal on the 6th, 9th and where necessary 12th post-operative days. Adult males were used throughout, their weights ranging from 1·5–3·5 kg. The median survival time of skin homografts transplanted to rabbits previously immunized with skin grafts from the same donor is 6·0 ± 0·6 days (Medawar, 1944).

Guinea-pigs

The donors were members of a closed pen-inbred colony of albino animals, whereas the recipients belonged to a highly inbred parti-coloured strain, members of which were known to accept skin homografts transplanted between them. Adult males were used throughout, their weights ranging from 500–750 g.

The skin grafting procedures employed have been described in full elsewhere (Billingham and Medawar, 1951).

Preparation of Donor Cells and Their Administration

Suspensions of homologous washed spleen cells, free from undissociated tissue fragments, were used as antigen in all three species. In addition, thymocytes were also used in the mouse, and whole blood, of which the effective element is known to be the leucocytes (Medawar, 1946*a*; Billingham, Brent and Medawar, 1956) was also used in the guinea-pigs.

With mice, the use of inbred strains made preservation of the spleen or thymus donors unnecessary. With rabbits and guinea-pigs, however, the donors had to be kept alive until the time of skin grafting; total splenectomy had therefore to be carried out.

Cell suspensions in Ringer-phosphate solution were prepared from the spleens by the procedure described in full by Billingham and Brent (1957). Suspensions comprising 80–90 per cent thymocytes were prepared by an essentially similar procedure from the organs removed from 6-week-old donors, the medium being Ringer-phosphate solution containing 0·6 per cent glucose. Cell counts were made with a haemocytometer.

With all the injections particular pains were taken to ensure that, so far as possible, the whole of the injection was administered through the intended route. In the event of failure to enter a vein cleanly, the animal was discarded.

Intravenous injections in mice were made in the tail vein, the volume of the inoculum being 0·5 ml. For subcutaneous injection a total volume of 0·5 ml. of suspension was divided equally between the skin of both flanks. Intradermal injections were also made bilaterally, the total volume administered being 0·1 ml. With these injections it was quite certain that most of the material was deposited between the panniculus and the corium rather than

intradermally in the usual sense. For the intramuscular injections the volume of the injection was reduced to 0·03 ml. and it was introduced, *viâ* a no. 30 gauge needle, into the belly of one of the thigh muscles. A small longitudinal skin incision was made beforehand to reveal the muscle. There was no observed tendency for the inoculum to leak out of the muscle on withdrawal of the needle, but this possibility cannot be entirely ruled out.

Guinea-pigs and rabbits received injections ranging from 3–5 ml. Intravenous injection of the guinea-pig was performed through the saphenous vein after its exposure by a small incision just above the "heel". Rabbits were injected through the marginal ear vein. In both species intradermal injections were made bilaterally at a number of scattered sites.

RESULTS

Mice

Tables I*a* and *b* show that with a dose of 5 million cells all 5 routes of administration tested were equally effective in eliciting transplantation immunity; this also held when 4 routes were tested with a dose of 250,000 cells. Five million spleen cells or thymocytes are clearly able to elicit a maximal response. Comparison of the dosages of spleen cells and thymocytes required to achieve complete destruction of the grafts in the majority of subjects suggests that there is little to choose between the antigenic potency of these two cell types. The skin graft test system is, as one would expect, the more sensitive of the two. It revealed that a dose of as little as 2000 spleen cells provoked a just perceptible immunity in 3 out of 5 animals. Whereas the skin graft test system could differentiate between various shades of immunity (by revealing the approximate percentage survival of the graft epidermis), the tumour test system (using the biological

TABLE I*a*.—*Percentage of Epithelial Survival in Test Grafts of CBA Skin 6 Days after Transplantation to A-strain Hosts Pre-treated with CBA Strain Spleen Cells by Different Routes*

Number of cells injected	Route of injection				
	Intravenous	Intraperitoneal	Subcutaneous	Intradermal	Intramuscular
5 million	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	25, 5, 0, 0, 0
250,000	50, 25, 0, 0, 0	10, 5, 5, 0, 0	0, 0, 0, 0, 0	—	5, 0, 0, 0, 0
50,000	—	100, 25, 10, 5, 0	—	—	—
10,000	—	75, 50, 25, 25, 10	—	—	—
2,000	—	100, 100, 90, 90, 25	—	—	—

TABLE I*b*.—*Percentage of Test Homografts of C57 BR/a Sarcoma Viable on Removal after 8 Days Residence in A Strain Mice Pre-treated with C57 BR/a Thymocytes*

Number of thymocytes injected	Route of injection		
	Intravenous (per cent)	Subcutaneous (per cent)	Intradermal (per cent)
50 million	0 (10)	0 (12)	—
5 „	0 (11)	0 (11)	0 (6)
1·58 „	0 (9)	17 (12)	—
500,000	36 (11)	40 (10)	—
158,000	100 (5)	70 (10)	—
50,000	83 (12)	82 (11)	100 (6)
5,000	—	100 (11)	—

Proportion of sarcoma grafts surviving on 8th day in A strain hosts immunized with tumour S778 = 0 per cent (12). Proportion of sarcoma grafts surviving in untreated A strain hosts on the 8th day = 93 per cent (14). The figures in brackets are the number of mice tested.

test of survival) merely revealed whether or not *some* viable cells remained in the graft at the time of autopsy.

The results obtained with spleen cells also show that the cell dose response curve is very flat over a wide dose range : from about 2000 to 5 million cells.

Rabbits

The injections of spleen cells into rabbits resulted in immunization by all three routes of administration, but consistently and powerfully only by the intradermal route (see Table II). Intraperitoneal injections gave irregular results in that the reactivity of half the subjects was completely unaltered.

TABLE II.—*Survival Times and Mode of Breakdown in Test Grafts Transplanted to Rabbits Pre-treated with Homologous Spleen Cells by Different Routes*

Number of cells injected (millions)	Route of injection		
	Intravenous	Intraperitoneal	Intradermal
300	<6,* <6*	9, 9, 6*	6*
240	8,* 6*	—	—
160	6,* 8*	6,* 10, 7*	<6,* <6,* 7*

* Indicates that graft breakdown was of the " immune " mode.

Guinea-pigs

The close similarity of the survival times of test grafts transplanted to 4 untreated recipients suggests that a reasonable uniformity of genetic disparity between donors and recipients has been obtained. The survival times of these control grafts are comparable with a M.S.T. of 9·2 days which can be computed from Sparrow's (1953, 1954) data for grafts exchanged between male guinea-pigs from heterogeneous laboratory stocks. With spleen cells it can be seen that all three routes tested were immunologically equivalent (Table III). With leucocytes (in the form of homologous citrated blood), too, there is nothing to choose between the intradermal and intravenous routes. No satisfactory explanation can be offered to account for the one animal which did not become immune after intravenous injection with homologous blood.

TABLE III.—*Survival Times and Mode of Breakdown in Test Grafts Transplanted to Guinea-pigs Pre-treated with Homologous Spleen Cells or Leucocytes by Different Routes*

Type of cell	Number of cells injected (millions)	Route of injection		
		Intravenous	Intraperitoneal	Intradermal
Spleen	90	<6,* <6*	—	6*
	70	—	<6,* 6*	<6*
	60	6*	<6*	<6*
Leucocytes	60	6*	—	—
	55	7*	—	—
	50	<6*	—	6,* <6*
	45	10	—	6*
	25	—	—	6*

Survival times of grafts on controls: 9, 9, 9, 10 days. * Indicates that graft breakdown was of the " immune " mode.

CONCLUSIONS AND DISCUSSION

The results presented here are consistent with the thesis that in mice and guinea-pigs all routes of administration of homologous cells tested are immunologically equivalent and elicit transplantation immunity consistently. However, this equivalence of routes is not found in rabbits. In contradistinction to massive dosages of whole blood, leucocytes or epidermal cells (Medawar, 1946*a* ; Billingham and Sparrow, 1955), spleen cells introduced intravenously in this species do elicit definite though not always powerful transplantation immunity. The intradermal route is here the most effective one, as other workers have found with other types of homologous cell, and the intraperitoneal route gives very irregular results indeed. Billingham and Sparrow (1955) failed to get immunity following two consecutive intraperitoneal injections of epidermal cells, each of which would have been sufficient to elicit transplantation immunity if given intradermally. The inconsistencies obtained with the intraperitoneal route in rabbits may be due to experimental shortcomings rather than to a failure of response on the part of some of the recipients. Stoufer and Paine (1957) have recently investigated the technique of intraperitoneal injection and have found that in the rabbit a surprisingly small proportion of injections are truly intraperitoneal ; many of the inocula find their way into the recipients' intestines even when considerable care is taken to avoid this.

The poor or even non-existent power of rabbit epidermal cells and leucocytes to elicit immunity when injected intravenously contrasts strongly with the powerful immunity which results from the intravenous injection of spleen cells. This anomalous situation may well be explicable in terms of the ultimate fates of these different types of cell. Homologous spleen cells introduced into the blood stream of very young mice are known to settle out electively in lymphoid tissue (Billingham and Brent, 1957), and intraperitoneally injected homologous bone marrow or spleen cells injected into irradiated adult mice show a similar " homing " instinct (Mitchison, 1956 ; Ford, Hamerton, Barnes and Loutit, 1956). The fate of homologous leucocytes or epidermal cells introduced into the blood stream of rabbits may well be different. They may be eliminated from the organism by a mechanism which does not involve any immunological response.

The great disparity between the efficacy of the intradermal and intravenous routes of immunization of rabbits with leucocytes has repeatedly been cited as evidence for the similarity of transplantation immunity and sensitization reactions of the delayed type (Medawar, 1946*b*, 1954 ; Burnet and Fenner, 1949 ; Mitchison, 1954). In the light of the present finding with spleen cells this evidence loses much of its weight.

SUMMARY

The equivalence of different routes of administration of suspensions of homologous cells for the elicitation of transplantation immunity has been compared in the three most common laboratory mammals.

In the mouse, where suspensions of spleen cells or thymocytes were used as the antigen, all five routes tested were equally effective over a wide dosage range. In the guinea-pig, too, in which spleen cells or leucocytes were employed, intravenous, intraperitoneal, or intradermal inoculations were equivalent in their efficacy. With the rabbit, however, spleen cells injected intradermally elicited

a more powerful immune response than when injected intravenously, and intraperitoneal injections gave irregular results. The finding that homologous spleen cells will immunize rabbits when administered intravenously is of interest since both leucocytes and epidermal cells are known to be ineffective when administered by this route.

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MINERAL REQUIREMENTS FOR GROWTH OF *STAPHYLOCOCCUS PYOGENES*. EFFECT OF SODIUM IONS

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SODIUM is often added to bacteriological media although there is little evidence that it is necessary for bacterial growth. Bacteria, like plants, are not entirely selective in their uptake of cations, and it is probable that absorption of cations and anions must proceed together to maintain a balance of charges. Possibly for this reason sodium is normally found in plant and bacterial ash, although it has not been found necessary for plants and at least some bacteria.

Using an ion exchange resin, we have previously shown that magnesium, calcium and potassium are necessary for the growth of *Staphylococcus pyogenes* in a complex medium (Shooter and Wyatt, 1955, 1956). This paper records the results of experiments designed to determine the effect of sodium on the growth of *Staph. pyogenes*.

MATERIALS AND METHODS

Cleaning of glassware

Glassware was cleaned as before (Shooter and Wyatt, 1956).

Reagents

Pyrex glassware was used. Metal-free water was prepared by passing distilled water down two columns containing the mixed ion exchange resin MB-3. Cations were added to the treated medium as the " Specpure " salts, NaCl, KCl, $MgSO_4.7H_2O$ and $CaCl_2$, purchased from Johnson Matthey.

Medium

The medium was made up at 4 times the final strength (1 per cent Lab Lemco, 1 per cent peptone and 0·5 per cent glucose) in 1 l. of metal-free water. For control broth 100 ml. of this were made up to 500 ml. and brought to pH 7·3. The remaining 900 ml. were run slowly down columns (2·5 cm. diam.) containing 1100 ml. of IRC-50 in the K cycle (the resin being treated with $KHCO_3$). The eluate was collected in a clean measuring cylinder, diluted with metal-free water and brought to pH 7·3 with redistilled HCl. For use the medium was placed in 8 ml. amounts in 6 × ¾ in. (15·2 × 1·9 cm.) tubes and made up to 10 ml. with reagents and water. When prepared it contained 0·022 mg. ions/l. Na^+ and 45 mg. ions/l. K^+.

Sodium measurements

Sodium was measured with an EEL flame photometer. The standard solutions were prepared in the treated medium at the same time and contained the same reagents (except the inoculum) as the test solutions.

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Inoculum

Strain " Terry " was used. Plate counts showed that approximately 2000 bacteria were added to each tube. There was no detectable addition of Na^+ to the medium by the inoculum.

Agar

Agar (2·5 g.) was dissolved in 200 ml. of the treated broth and shaken intermittently for 1 hr. at 80° with 100 ml. of IRC-50 (K) in a flask. The agar broth was then decanted and brought to pH 7·3. For use it was dispensed in 12 ml. amounts and made up to 15 ml. with reagents and water.

RESULTS

The treated broth did not support growth by itself, or after the addition of 0·08 mg. ions/l. Mg^{2+} or 0·05 mg. ions/l. Ca^{2+} or up to 20 mg. ions/l. Na^+.

Effect of magnesium and calcium on growth

When Mg^{2+} and Ca^{2+} were added together to the treated broth there was good growth (Table I) although less than had been obtained previously in the presence of 70 mg. ions/l. Na^+ (Shooter and Wyatt, 1955).

TABLE I.—*Effect on the Growth of* Staph. pyogenes *at* 18 *hr. of the Addition of* Mg^{2+} *and* Ca^{2+} *to Medium Containing Low* Na^+ *and High* K^+ *Concentration*

(Results expressed as a percentage of control growth)

		mg. ions/l. Mg^{2+} added						
		0·01	0·02	0·04	0·08	0·2	0·4	0·8
mg. ions/l. Ca^{2+} added	0·005	6	28	—	37	—	—	—
	0·05	27	—	31	—	48	—	—
	0·1	—	—	42	48	53	57	59
	0·25	—	—	52	53	55	59	58
	0·5	—	—	52	57	55	58	59

Broth batch N.1.

Effect of sodium and potassium on growth

The addition of Na^+ with Ca^{2+} and Mg^{2+} depressed growth when Ca^{2+} was present in sub-optimal amounts, but had no effect with higher concentrations of Ca^{2+} (Figure). In a preliminary experiment (Wyatt, 1957) with a less rigorously treated broth containing 62 mg. ions/l. K^+ and 0·115 mg. ions/l. Na^+ (as compared with 45 mg. ions/l. K^+ and 0·022 mg. ions/l. Na^+ in the treated broth) growth was depressed by the addition of Na^+, and the addition of Ca^{2+} was without effect. In the main experiment the depression of growth at low Ca^{2+} levels was not remedied by increasing the K^+ concentration to 65 mg. ions/l., and at the lowest Ca^{2+} concentration the increase in K^+ led to a further decrease in growth (Figure).

Uptake of sodium

Table II shows that at varying Mg^{2+} and Ca^{2+} concentrations and different Na^+ levels there was no uptake of Na^+. The fact that there was no uptake at Na^+ levels which suppressed growth suggested that the depressive effect was extra-cellular.

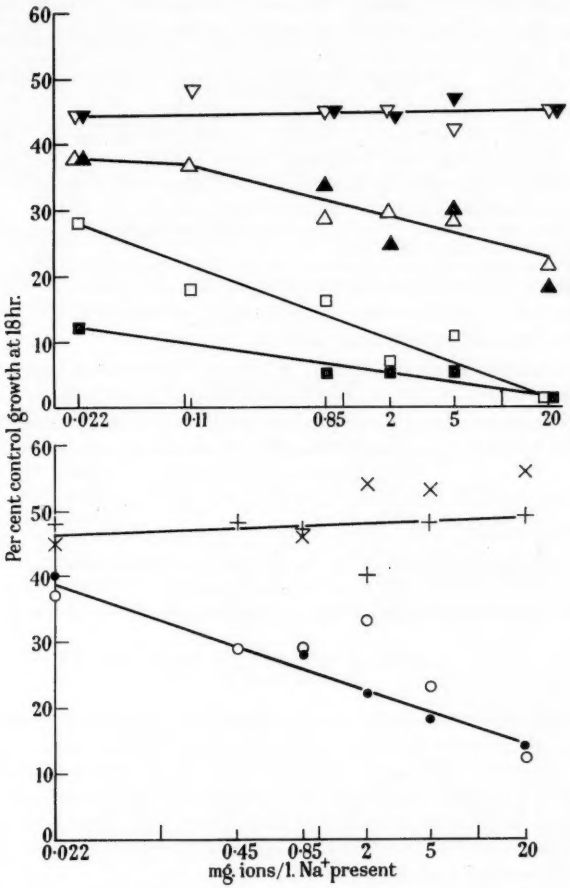

FIGURE.—The effect of the addition of Na+ and K+ on the growth of *Staph. pyogenes* at 18hr. in the presence of additions of Mg^{2+} and Ca^{2+}. Broth batch N.1.

	mg. ions/l.		
	Magnesium added	Calcium added	Potassium present
□ . .	0·02	0·005	45
■ . .	0·02	0·005	65
△ . .	0·02	0·05	45
▲ . .	0·02	0·05	65
▽ . .	0·02	0·5	45
▼ . .	0·02	0·5	65
○ . .	0·08	0·005	45
● . .	0·08	0·005	65
+ . .	0·2	0·05	45
× . .	0·2	0·05	65

TABLE II.—*Uptake of Na^+ at 18 hr. by* Staph. pyogenes

Mg^{2+} mg. ions/l. added	Ca^{2+} mg. ions/l. added	Na^+ mg. ions/l. (uncorrected readings)					
		Standard	Residual	Standard	Residual	Standard	Residual
0·02	0·005	0·018	0·025	0·106	0·107	0·85	0·85
0·02	0·05	0·019	0·025	0·103	0·109	0·85	0·86
0·02	0·5	0·020	0·023	0·114	0·114	0·85	0·86
0·08	0·005	0·019	0·021	0·45	—	0·83	0·84
0·2	0·05	0·019	0·020	0·45	0·45	0·85	0·85

Broth batch N.1.

Standard: Each standard was made up in the treated broth, together with the Ca^{2+} and Mg^{2+} shown, but was not inoculated or incubated.

Residual: The bacteria were spun down for 20 min. at 1800 *g* and the Na^+ in the supernatant medium was measured with an EEL flame photometer.

Characteristics of growth in deficient medium

With low concentrations of Mg^{2+} and Ca^{2+} in the treated medium growth was confined to the bottom of the tube. On shaking thin strands of bacteria swirled up and twisted to give the appearance of rope. An even suspension could only be made with a syringe. There was no evidence of chain formation in the aggregates.

Growth on solid medium

There was little growth after 36 hr. on treated agar broth. With adequate Mg^{2+} and Ca^{2+}, but not Na^+, there was good growth. The addition of Na^+ caused no apparent change in the appearance of the colonies.

DISCUSSION

Eddy and Hinshelwood (1950) could detect no appreciable uptake of radioactive sodium by *Bacterium lactis aerogenes*, and found that sodium was not necessary for growth. Omission of sodium from media used for the assay of amino acids and vitamins increased the growth of lactic acid bacteria in all but one assay (Sirny, Braekkan, Klungsoyr and Elvehjem, 1954) and sodium was not necessary for the growth of *Clostridium welchii* although there was chain formation in its absence (Shankar and Bard, 1952).

Our study shows that sodium is not necessary for the growth of *Staph. pyogenes* in liquid or on solid media, and that there was no detectable uptake of sodium from the medium, measured directly without washing the bacteria. Mitchell and Moyle (1956), using a thick suspension of bacteria in sodium chloride, showed that *Staph. pyogenes* was readily permeable to sodium. Our method would not reveal the slight uptake from the medium necessary for equilibrium, but shows that there is no accumulation of sodium even when it is present in very low concentration.

As few of their specific functions are known, the essentiality of cations must be determined from the growth responses of the micro-organism (see Nicholas, 1953). If however an element is removed from the basic culture medium and its subsequent addition increases growth, then accumulation of this element within its stimulating range will be further evidence that it is essential. There may be no accumulation if the element is required extra-cellularly only. Similarly, if

an element is removed and subsequent addition does not increase growth, then the absence of accumulation will be further evidence that it is not essential.

SUMMARY

The sodium content of nutrient broth was lowered to 0·022 mg. ions/l. by passage through an ion exchange resin. Sodium was not found to be necessary for the growth of *Staph. pyogenes* and there was no detectable uptake of sodium during growth. Addition of sodium in some circumstances depressed growth. Sodium was not necessary for growth on agar.

We would like to express our thanks to the Treasurer and Board of Governors of St. Bartholomew's Hospital for a grant which enabled this work to be carried out.

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VOL. XXXVIII, No. 4 AUGUST, 1957

CONTENTS

PAGE

THRELFALL, C. J. AND STONER, H. B.—STUDIES ON THE MECHANISM OF SHOCK. THE EFFECT OF LIMB ISCHAEMIA ON THE PHOSPHATES OF MUSCLE . . 339

KIRSCHNER, L., MAGUIRE, T. AND BERTAUD, W. S.—FURTHER EVIDENCE OF THE ANTILEPTOSPIRAL EFFECT OF MILK: ELECTRON MICROSCOPIC STUDIES . . 357

CINADER, B.—THE DANYSZ EFFECT: AN INVESTIGATION OF THE REACTION BETWEEN AN ENZYME AND ITS ANTIBODY 362

TAVERNE, JANICE.—IMMUNE-ADHERENCE OF BACTERIOPHAGE T2 . . . 377

COHEN, A. AND SMITH, WILSON.—THE HETEROGENEITY OF INFECTIVE VIRUS PARTICLES WITHIN A SINGLE INFLUENZA VIRUS STRAIN 385

KELLY, BARBARA AND PEREIRA, H. G.—ACTIONS OF ADENOVIRUS TYPES 5 AND 7 IN CULTURES OF RABBIT KIDNEY TISSUE 396

PORTER, K. A.—EFFECT OF HOMOLOGOUS BONE MARROW INJECTIONS IN X-IRRADIATED RABBITS 401

BATTEN, J. C. AND MCCUNE, R. M. JR.—THE INFLUENCE OF CORTICOTROPHIN AND CERTAIN CORTICOSTEROIDS ON POPULATIONS OF *Mycobacterium tuberculosis* IN TISSUES OF MICE 413

BATTEN, J. C. AND MCCUNE, R. M. JR.—THE INFLUENCE OF CORTICOTROPHIN AND CORTISONE WITH ANTITUBERCULOUS DRUGS ON POPULATIONS OF *Mycobacterium tuberculosis* IN TISSUES OF MICE 424

HEPTINSTALL, R. H.—THE EFFECTS OF HIGH BLOOD CHOLESTEROL ON THE PULMONARY ARTERIAL CHANGES PRODUCED IN RABBITS BY THE INJECTION OF BLOOD CLOT 438

WILHELM, D. L., MILL, P. J. AND MILES, A. A.—ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION. III. FURTHER OBSERVATIONS ON THE PERMEABILITY FACTOR AND ITS INHIBITOR IN GUINEA-PIG SERUM 446

STEWART, P. B. AND BLISS, J. Q.—THE PERMEABILITY-INCREASING FACTOR IN DILUTED HUMAN PLASMA 462

BILLINGHAM, R. E., BRENT, L. AND MITCHISON, N. A.—THE ROUTE OF IMMUNIZATION IN TRANSPLANTATION IMMUNITY 467

SHOOTER, R. A. AND WYATT, H. V.—MINERAL REQUIREMENTS FOR GROWTH OF *Staphylococcus pyogenes*. EFFECT OF SODIUM IONS 473

SURVEY OF PAPERS i

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